Remarks

Claims 1-7, 9-11, 17, 20, 25, 28, 30 and 39-43 are pending. Claims 1-3, 6, 7, 9-11, 20, 25, 28, 30, and 39-43 have been amended. Applicants believe these amendments attend to all the cited objections, as well as rejections based on assertions of indefiniteness. New claims 44-47 have been added.

Claim Rejections - 35 USC 112 Written Description and Enablement Requirements

Claims 1-7, 9-11, 17, 20, 25, 28, 30 and 39-43 stand rejected based on the contention that they are supported by an inadequate written description and/or are not enabled.

In response, Applicants point out that there is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed (MPEP 2163(II)). Moreover, it is established that an Applicant may show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics (MPEP(II)(3)(a)). In view of this, Applicants respectfully point out the following in connection with the instant application and Items 8-30 of the present Office Action.

Applicants submit that one skilled in the art would recognize that alanyl aminopeptidases is a metal-dependent integral membrane protease and that his enzyme (as well as enzymes having a similar substrate specificity) belong to the M1 family of the MA group of peptidases, also referred to in the art as gluzincins. Accordingly, it will be recognized that the genus "inhibitors of alanyl aminopeptidase" (APN) includes compounds that are capable of specifically inhibiting the enzyme activity of alanyl aminopeptidase, as well as other enzymes having a similar substrate specificity.

In this regard, it is established in the art that the presently recited inhibitors share common characteristics that unite them into a proper genus. For example, it is recognized by those skilled in the art that a common characteristic feature of these inhibitors is their affinity of the active site of alanyl aminopeptidase or enzymes having a similar substrate specificity.

Specifically, the catalytic domain of such enzymes is characterized by a zinc ion, a zinc binding motif with the sequence HEXXH-(X18)-E and the peptidase motif GXMEN. The zinc is coordinated by two histidine residues and one glutamate residue C-terminal to the second histidine. The amino acid residues which are responsible for the binding of inhibitors in the active site of the alanyl aminopeptidase are E355, H388, E389, H392, E411 and Y477. This is applicable for all inhibitors of alanyl aminopeptidase. Further, with respect to their inhibitory function, it is known that a mutation of glutamic acid 355 in an aminopeptidase conserved region (GXMEN or GAMEN motif) leads to an inactive enzyme, which demonstrates that this glutamic acid belongs to the anionic binding site in APN and interacts with the N-terminal α-amino group of its substrate. Thus, these common characteristics of the interaction between inhibitors of alanyl aminopeptidase and enzymes having similar substrate specificities permit classification of the inhibitors into a proper genus based on their known structural / functional interactions with such enzymes.

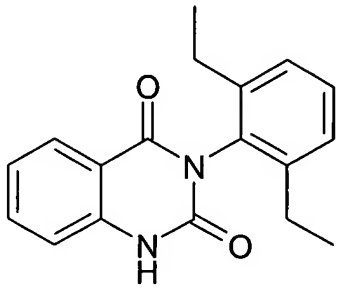
Notwithstanding the foregoing, those skilled in the art will recognize that inhibitors of alanyl aminopeptidase may be classified into several different structural classes, depending upon recognized structural elements of such inhibitors and their known catalytic zinc-binding domain function. For example, the inhibitors can be divided into naturally occurring or synthetic inhibitors, either of which bind directly to the catalytic site of the enzyme. The latter can be divided into, for example, ß-amino thiols inhibitors, α-amino aldehydes inhibitors, α-aminophosphonates inhibitors, 4-amino-L-proline inhibitors, α-aminoboronic acid inhibitors.

The principal approaches taken for the design of synthetic APN inhibitors is the recognized substrate-based design method wherein, in general, peptidomimetics that incorporate zinc binding groups and P and/or P' side chains to interact with the enzyme subsites are the most common structural features. Hence, the functional relationship for any alanyl aminopeptidase inhibitors is their ability to inhibit the enzyme activity of alanyl aminopeptidase and other enzymes having a similar substrate specificity, independent of their chemical diversity. Examples of peptidic and non-peptidic APN inhibitors have proliferated the literature over the last several years. In support of this, Applicants submit herewith the reference Xu, W. et al.: "Progress in the Development of Aminopeptidase N (APN/CD13) Inhibitors" in Curr. Med. Chem. – Anti-cancer Agents, 2005, 5, 281 – 301.

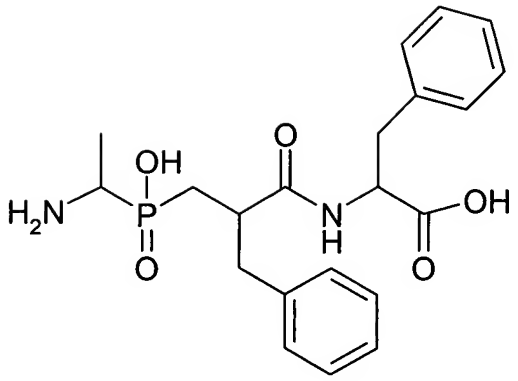
As for naturally occurring inhibitors, examples of these include, for example, actinonin, amastatin, bestatin, phebestin, probestin, leuhistin, and others that would be recognized by those skilled in the art. The inhibitor phebestin used in the examples of the present patent application is structurally similar to the inhibitors bestatin and probestin.

With respect to certain compounds the Examiner contends are not disclosed or known in the prior art, Applicants respectfully disagree. Each of these are well known to one skilled in the art of aminopeptidase inhibitors.

Specifically, the inhibitor termed PAQ-22 is a synthetic inhibitor, representing an α-aminophosphonate inhibitor. The chemical name for RB3014 is 3-(2,6-diethylphenyl-)quinazoline-2,4(1H,3H)-dione; its molecular weight is 294.3 and its formula is $C_{18}H_{18}N_2O_2$. PAQ-22 is a specific inhibitor of the cytosolic alanyl aminopeptidase (which is an enzyme having a similar substrate specificity). The inhibitor inhibits the membrane-bound aminopeptidase as well as the cytosolic amino peptidase in a non-competitive manner and is based on a homophthalimide structure. It is known to those skilled in the art to have the following structure:



RB3014 is also a well recognized inhibitor and one skilled in this art would be familiar with its structure accordingly. The chemical name for RB3014 is 2{3-[(1-amino-ethyl-)hydroxy-phosphinoyl-]2-benzyl-propionylamino-}-3-phenyl-propionic acid; its molecular weight is 418.4 and its formula is $C_{21}H_{27}N_2O_5P$. It is known by those skilled in the art to have the following structure:



Likewise, MR 387 is well known to those skilled in the art to exist in two different forms, namely MR 387 A and MR 387 B. MR 387 A is AHPA-Val-Pro-Hyp (2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-prolyl-(trans-4-hydroxy-L-proline). MR 387 B is AHPA-Val-Pro-Pro (2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-prolyl-L-proline. In furtherance of the established recognition of this by one skilled in the art, Applicants submit the reference of Bauvois B. et al. (2006) Medicinal Research Reviews, Vol. 26, No. 1, pp88 -130, enclosed herewith.

In view of the foregoing, Applicants respectfully submit that, since it is axiomatic that one should not provide a description in the specification of that which is known to one skilled in the art, an adequate written description of inhibitors has been provided such that one skilled in the art would conclude that Applicants were in possession of the invention at the time the present application was filed.

With regard to the phrase "enzyme having similar substrate specificity" the reference of Albiston A.L. et al. "Membrane bound members of the M1 family: more than aminopeptidase" in Protein and Peptide Letters, Vol. 11, No. 5, 491 – 500 (2004) is submitted herewith. As evidenced by this reference, Applicants respectfully submit that the following aminopeptidases belong in the genus of enzymes which have similar substrate specificity relative to APN:

- Aminopeptidase N (APN);
- Aminopeptidase A (APA);
- Thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE);
- Adipocyte-derived leucine aminopeptidase (A-LAP);

- Insulin-regulated aminopeptidase (IRAP);
- Aminopeptidase B (APB);
- Puromycin-sensitive (cytosolic) aminopeptidase (PSA);
- Leukotriene A4 hydrolase (LTA4H); and
- Leucocto-derived arginine aminopeptidase (L-RAP).

Moreover, one skilled in the art would recognize that enzymes having APN-analogous activity have two conserved sequence elements, namely the HEXXH-(X18)-E consensus zinc-binding motif and the GXMEN exopeptidase motif in the catalytic domain, as well as common substrate activity, and therefore common inhibitors.

In more detail, bestatin is capable of inhibiting APN activity, as well the activity of enzymes having APN-analogous enzymatic activity, namely Puromycin-sensitive (cytosolic) aminopeptidase (PSA) and Leukotriene A4 hydrolase (LTA4H). Similarly, PAQ-22 is capable of inhibiting APN, as well as Puromycin-sensitive (cytosolic) aminopeptidase (PSA).

Specificity of the inhibitors is defined by the IC_{50}-value (see for example, the enclosed reference of Xu, W. et al.).

Applicants further point out that methods are known to those skilled in the art for testing the activity of a broad range of alanyl aminopeptidase inhibitor compounds. In connection with this, it is well known that autoimmune diseases or allergies are diseases with an imbalanced immune response mainly due to a loss of functional suppression by regulatory T cells. Regulatory T cells are one of the most potent producers of the immunosuppressive cytokine TGF-β1. Therefore, the activation of regulatory T cells and the stimulation of TGF-β1 by administering alanyl aminopeptidase inhibitors according to the method of the invention is a reasoned approach for treatment of these immune-mediated diseases.

With respect to the enablement requirement, Applicants respectfully submit that to ascertain the potency of alanyl aminopeptidase inhibitors in activation of regulatory T cells, the inventors have employed the following methods: Measurement of the effects of alanyl aminopeptidase inhibitors on the activity of alanyl aminopeptidase using purified aminopeptidase N; Measurement of the effects of alanyl aminopeptidase inhibitors on the activity

of alanyl aminopeptidase using purified cytosolic alanyl aminopeptidase; Measurement of the effects of alanyl aminopeptidase inhibitors on the activity of cell-bound alanyl aminopeptidases using different cell systems; Measurement of the effects of alanyl aminopeptidase inhibitors on the induction of TGF-β1-mRNA of purified regulatory T cells from human blood; Measurement of the effects of alanyl aminopeptidase inhibitors on the induction of TGF-β1-mRNA of purified regulatory T cells from human blood; Measurement of the effects of alanyl aminopeptidase inhibitors on the induction of cell membrane-bound TGF-β1 of purified regulatory T cells from human blood; and, Measurement of the effects of alanyl aminopeptidase inhibitors on the induction of TGF-β1 in supernatants of purified regulatory T cells from human blood.

Applicants further submit that one skilled in the art at the time the present application was filed would appreciate that the course of diseases having an autoimmune pathogenesis, as for example diabetes mellitus type I or multiple sclerosis, is based on the activation and proliferation of autoreactive immune cells, i.e. immune cells directed against self-antigens, and in particular of autoreactive T-lymphocytes. Similar mechanisms are responsible for organ rejection after transplantation, but with the difference that, in the latter case, it is not autoantigens but foreign antigens of the donor organ that are primarily responsible for development of the adverse immune response. In both cases, i.e., in cases of autoimmune diseases and in cases of graft rejection reactions, there is an undesired break of tolerance of the immune system towards these antigens. A similar explanation is valid for the excessive immune response in allergic diseases. It would be known to one skilled in the art that such a "tolerance" of the immune system is maintained in by actively suppressing autoreactive T-lymphocytes. This is achieved by a specific suppression of a T-cell population, i.e. the regulatory T-cells, in particular CD4+CD25+T-cells. When the aforementioned diseases are present, the regulatory T-cells in part lose their ability to suppress inflammation which leads to a worsening of disease. Accordingly, the present invention provides a novel approach for the activation of regulatory T-cells and the induction of the immunosuppressive agent TGF-ß1 through inhibition of alanyl aminopeptidase and enzymes having a similar substrate activity. Thus, Applicants respectfully submit that the inhibition of alanyl aminopeptidase and enzymes having a similar substrate activity according to the present invention results in an immunosuppressive effect, and the use of one or of several such inhibitors for the induction of TGF-ß1 and the expression of TGF-ß1 in

regulatory T-cells cells is accordingly adequate enable the entire scope of the now pending claims. The Examiner is therefore courteously requested to remove the rejections imposed under the written description and enablement requirements of 35 USC 112.

Claim Rejections under 35 USC 102(b)

In connection with the claim rejections under 35 USC 102(b) as anticipated by Ansorge et al., (WO 01/89569) Applicants point out that the present claims have been amended to recite that the compositions are for use in patients in need of induction of the production of TGF-β1 and of the expression of TGF-β1 in and/or on regulatory T cells. There is no such disclosure in the aforementioned publication of Ansorge et al. The Examiner is respectfully requested to remove the stated rejections.

In connection with the rejections of the claims under 35 USC 102(b) as anticipated by Andrulis (WO 95/04533), Applicants have amended the claims to cancel reference to thalidomide. Therefore, the reference of Andrulis cannot be deemed to anticipate these claims. The Examiner is respectfully requested to remove the stated rejections.

Claim Rejections under 35 USC 102(e)

With respect to the rejections of the claims under 102(e), and as the Examiner has pointed out, all of the cited US patent applications have a common inventor with the instant application. Thus, Applicants respectfully request the Examiner to hold this rejection in abeyance until such time as the instant application is deemed otherwise allowable, after which Applicants intend to submit a declaration in support of the patentability of the present claims.

Double Patenting

With respect to the rejections based on the provisional obviousness-type double patenting rejections, Applicants respectfully request the Examiner to hold this rejection in abeyance until such time as the present application is deemed otherwise allowable, after which time Applicants will submit any necessary terminal disclaimer(s) or distinguish the present invention from the cited applications on the merits.

Conclusion

In view of the foregoing, Applicants respectfully submit that all the pending claims are now in condition for allowance. The Examiner is thus requested to allow all the claims. Applicants herewith request a three-month extension of time to file this response. A check for the required fee is enclosed. Any additional fees due it may be charged (or any overpayments credited) to Deposit Account no. 08-2442.

Respectfully submitted,
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Aminopeptidase-N/CD13 (EC 3.4.11.2) Inhibitors: Chemistry, Biological Evaluations, and Therapeutic Prospects

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Abstract: Aminopeptidase N (APN)/CD13 (EC 3.4.11.2) is a transmembrane protease present in a wide variety of human tissues and cell types (endothelial, epithelial, fibroblast, leukocyte). APN/CD13 expression is dysregulated in inflammatory diseases and in cancers (solid and hematologic tumors). APN/CD13 serves as a receptor for coronaviruses. Natural and synthetic inhibitors of APN activity have been characterized. These inhibitors have revealed that APN is able to modulate bioactive peptide responses (pain management, vasopressin release) and to influence immune functions and major biological events (cell proliferation, secretion, invasion, angiogenesis). Therefore, inhibition of APN/CD13 may lead to the development of anti-cancer and anti-inflammatory drugs. This review provides an update on the biological and pharmacological profiles of known natural and synthetic APN inhibitors. Current status on their potential use as therapeutic agents is discussed with regard to toxicity and specificity. Med Res Rev, 26, No. 1, 88–130, 2006

Key words: aminopeptidase; ectoenzyme; natural inhibitor; synthetic inhibitor; bestatin; cancer; inflammation

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1. INTRODUCTION

Aminopeptidase N (EC 3.4.11.2, APN) is a metallo-dependent integral membrane protease.[1] The enzyme belongs to the M1 family of the MA clan of peptidases[2] also called gluzincins.[3] Aminopeptidase N consists of 967 amino acids with a short N-terminal cytoplasmic domain, a single transmembrane part, and a large cellular ectodomain containing the active site.[4] This enzyme was first isolated in 1963 by Pfleiderer and Celliers from pig kidney[5] and is known under several different names (alanine aminopeptidase; microsomal aminopeptidase; microsomal leucine aminopeptidase aminopeptidase M; amino oligopeptidase; GP 150). In the last few years, certain surface molecules identified as cluster differentiation (CD) antigens were found to be identical to some membrane proteins. Thus, CD13 is identical to APN.[6,7] Soluble APN is detectable in plasma/serum and urine[8–11] but the mechanism of release of membrane APN remains unknown.

Membrane-bound APN/CD13 is widely distributed outside the hematopoietic system (epithelial-, endothelial-, fibroblast-cell types) with main sources being brush border membranes of kidney proximal tubule cells and enterocytes, and in the hematopoietic compartment is not confined to a particular lineage.[1,12,13] APN/CD13 is predominantly expressed on stem cells and on cells of the granulocytic and monocytic lineages at distinct stages of differentiation and is therefore considered as a marker of differentiation.[14,15]

Dysregulated expression of membrane and/or soluble forms of APN/CD13 is observed in many diseases. Compiled observations indicate enhanced APN levels in tumor cells such as melanoma,[16,17] renal,[18] pancreas,[19] colon,[20] prostate,[21] gastric,[22] and thyroid[23] cancers. Tumor-infiltrating T cells in renal and lung cancers are CD13-positive.[24,25] APN activity is elevated in plasma and effusions of cancer patients.[11] APN activity on neutrophils from patients affected by a rare adrenal gland tumor, adrenal pheochromocytoma, is significantly increased as compared with healthy controls.[26] CD13 is overexpressed in acute and chronic myeloid leukemias[1,12,27–29] and in anaplastic large cell lymphomas.[30,31] Overexpression of APN/CD13 in T lymphocytes or neutrophils occurs in several inflammatory diseases (chronic pain, various forms of joint effusions, rheumatoid arthritis, multiple sclerosis, systemic sclerosis, systemic lupus erythematosus, polymyositis/dermatomyosytis, pulmonary sarcoidosis).[32–39]

APN/CD13 may be therefore considered as a useful clinical marker. Whether this protease critically contributes to the pathological behavior remains however unknown. In this review, we briefly summarise knowledge on the structure and the mechanisms of cleavage of APN/CD13 to integrate current knowledge in natural and synthetic APN inhibitors. The reader is referred to excellent reviews for the characteristics of APN/CD13 and substrate specificity.[25,40–45] Various aspects on the roles of APN/CD13 are reviewed here in the context of the *in vitro* and *in vivo* use of certain APN inhibitors.

2. AMINOPEPTIDASE N/CD13

APN is anchored to the plasma membrane, *via* an uncleaved signal sequence, by the C-terminus (type II) facing extracellularly.[1] Membrane APN/CD13 is found as a dimer of two non covalently associated subunits with a relative molecular mass of 160 kDa (Fig. 1A).[40,41,43] The human CD13 gene, cloned in 1989[6] and subsequently mapped to chromosome 15 q25-26,[46] possesses two promoters (Fig. 1B).[46–51]

The cDNA sequence reveals the presence of the amino acid sequence His-Glu-Xaa-Xaa-His which is a Zn^{++} binding motif found in one class of metallo-peptidases.[3] Site-directed mutagenesis indicates that extracellular cysteines in the molecule confer correct structure and consequently enzymatic activity and surface expression of APN.[52] Mutation of glutamic acid 355 in an aminopeptidase conserved region (the GAMEN motif) leads to an inactive enzyme[53] indicating that

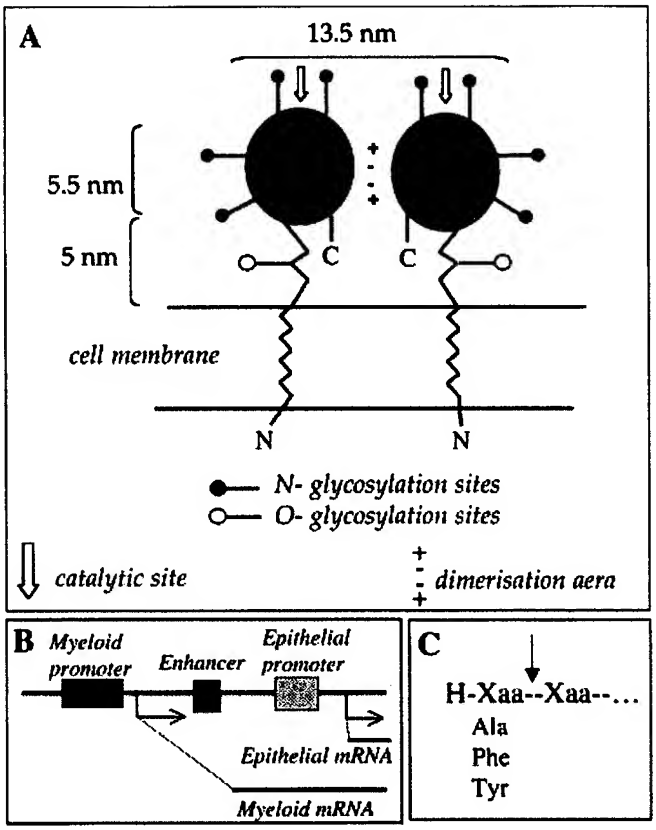


Figure 1. Schematic diagrams showing protein (A) and promoter (B) structures of APN/CD13. The enzyme is a dimer of two non covalently associated monomers. The gene is controlled by two promoters, with an epithelial promoter and a myeloid promoter (in the hematopoietic system). (C) substrate specificity. The arrow indicates the bond cleaved.

this glutamic acid belongs to the anionic binding site in APN and interacts with the N-terminal α-amino group of the substrate. APN/CD13 cleaves preferentially neutral amino acids (with the exception of proline) (Fig. 1C) from the unsubstituted N-terminus of oligopeptides.[1,12] Biologically active peptide substrates cleaved by APN/CD13 are neuropeptides (Met- and Leu-enkephalins, neurokinin A, Met-lys-bradykinin, and endorphins such as spinorphin),[41,54–59] vasoactive peptides (kallidin, somatostatin, and angiotensins)[60–67] and chemotactic peptides (monocyte chemotactic protein//MCP-1 and N-formyl methionine leucine phenylalanine/f-MLP).[40,68]

Apart from its hydrolytic ability, APN serves as a receptor for coronaviruses.[69–72] In humans, the 229E corona virus uses APN to enter alveolar cells and establish an upper respiratory tract infection.[72]

3. APN/CD13 INHIBITORS

Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) are natural peptides capable of inhibiting APN in micromolar concentrations.[73] Similarly, elevated concentrations of leucine, proline, L-alanine, L-arginine, L-glutamine, L-methionine, as well as divalent cations (Co^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, Ni^{2+}) inhibit APN activity.[40] (for review) Moreover, molecules with a broad spectrum of action such as KCN, NaN_3,

ammonium oxalate, *N*-ethyl-maleimide, and 8-hydroxyquinoline inhibit APN/CD13.[40] (for review) APN activity is also inhibited by puromycin (**1**),[74,75] lapstatin (**2**),[76] some *N*-phenylphthalimide derivatives such as compound **3**,[77–80] several *N*-phenylhomophthalimide derivatives like PIQ-22 (**4**)[77,78] which has later been described as a rather puromycin-sensitive aminopeptidase (PSA) inhibitor by the same group,[80–83] phosphinate dipeptide analogues illustrated by hPheP[CH_2]Tyr (**5**),[84] pseudoglutamyl aminophosphinic peptides such as GluΨ(PO_2CH_2)Leu-Ala (**6**),[85] several variously substituted 3-amino-2-oxobutyramide exemplified by compound **7**,[86] α-aminoboronic derivatives such as the benzyl derivative **8**,[87] or α-aminobenzaldehydes illustrated by (*S*) 2-amino-5-methylpentanal (**9**).[88] An eclectic set of compounds has been described and used for the biochemical characterization or/and inhibition of other proteases—e.g.: urokinase-type plasminogen activator, dipeptidylpeptidase IV (DPPIV/CD26), or other different aminopeptidases including human enkephalin degrading aminopeptidase (HEDA), cytosolic leucine aminopeptidase (LAPc), glutamyl aminopeptidase (APA), and arginyl aminopeptidase (AP-B). In this context, it is also worth mentioning two systematic studies devoted to hydroxylated naturally occurring flavonoids such as baicalein (**10**), apigenin (**11**), or myricetin (**12**) and related compounds which, aside their activity on neutral endopeptidase (NEP/CD10) or angiotensin-converting enzyme (ACE/CD143), exhibited a significant *in vitro* inhibitory effect toward APN.[89,90] Formulas, Ki, IC_{50} or inhibition percentages of enzymes for compounds **1–12** are depicted in Figure 2. Two recent publications describing either the irreversible inhibition of both APN/CD13 and DPP IV/CD26 enzymatic activities by aqueous extracts of a *Cistus incanus* L.[91] or ACE, NEP, and APN inhibition by extracts of *Epilobium angustifolium*[92] deserve also quotation.

Although the borderline is not easy to position, leaving out the above-mentioned studies dealing with non-specific compounds targeting other enzymes and, incidentally, revealing an inhibitory activity on APN, we have chosen to focus the present review on the data tightly dedicated to natural and synthetic inhibitors of APN/CD13 itself.

A. Naturally Occurring APN/CD13 Inhibitors

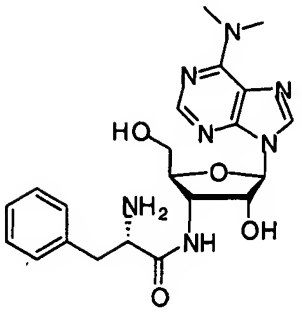
The most widely used among the naturally occuring APN/CD13 inhibitors are microorganism-produced and have been purified from microbial culture filtrates. A large part of them are generated by bacteria belonging to the order *Actinomycetales*, especially of the genera *Streptomyces*:

1. Actinonin

(2*R*)-N^4-hydroxy-N^1-[(1*S*)-1-[[2*S*)-2-(hydroxymethyl)-1-pyrrolidinyl]carbonyl]-2-methylpropyl]-2-pentylbutanediamide (**13**) was first isolated by R. Green and R. Bhagwan Singh from a Malayan strain of *Actynomycetes*. This compound was then listed as *Streptomyces* Cutter *C/2* (N.C.I.B. 8845).[93] About 20 years later, actinonin was also obtained from another strain referenced MG848-hF6 and its inhibition against APN was found to be competitive with the substrate.[94] The structural study and the chemical synthesis of **13** and some analogues have aroused numerous works[95–102] completed by a structure–activity relationship investigation dealing with anti bacterial properties observed in this actinonin series.[103]

2. AHPA-Val

(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valine) (**14**)) and two closely related derivatives: *AHPA-Val-Pro-Hyp* (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-prolyl-(*trans*-4-hydroxy-L-proline) (MR387A) (**15**) and *AHPA-Val-Pro-Pro* (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-prolyl-L-proline) (MR387B) (**16**) were obtained from the culture broth of *Streptomyces neyagawaensis* SL-387.[104–106] The preparation of several novel synthetic AHPA derivatives (exemplified by **17**) bearing, for most of them, heterocyclic moieties and exhibiting



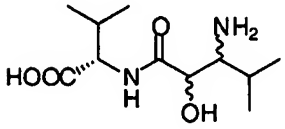
Puromycin (1)

IC_{50} APM from rat blood plasma : 60 μM[67]

IC_{50} APN : 50 μM, IC_{50} AAP-S : 0.6 μM μM[75]

67% inhibition of APN from human seminal plasma at 100 μM[74]

IC_{50} APN : 4.8 μM, IC_{50} PSA : 0.6 μM[83]



Lapstatin (2)
(The absolute configuration is not completely elucidated)

IC_{50} APM from hog kidney : 203 μM[76]

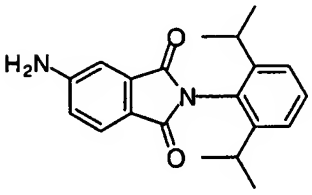
IC_{50} APN from egg white : > 122 μM[76]

IC_{50} LAP from porcine kidney : 41 μM[76]

IC_{50} LAP from *Streptomyces rimosus*: 2.4 μM[76]

IC_{50} LAP from *Aeromonas proteolytica*: 0.3 μM[76]

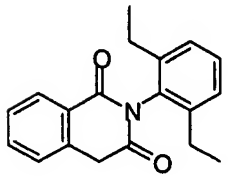
IC_{50} API from *Streptomyces griseus*: 0.85 μM[76]



3

IC_{50} APN : 5.4 μg/mL, IC_{50} DPP-IV : 14.1 μg/mL, WI-38 : 17.4 μg/mL[77,80]

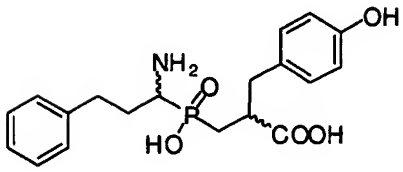
IC_{50} APN : 16.8 μM, IC_{50} DPP-IV : 251.2 μM[79]



PIQ-22 (4)

IC_{50} APN : 0.12 μg/mL, IC_{50} DPP-IV : 14.1 μg/mL[77,80]

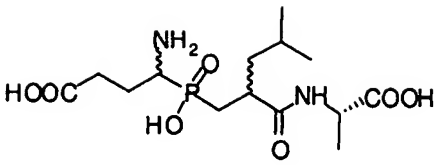
Inactive towards LAP, DPP-IV, Trypsin and Chymotrypsin[80,82]



hPheP[CH_2]Tyr (5)
(mixture of four diastereoisomers)

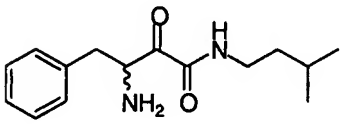
K_i APN : 36 nM, K_i LAP : 67nM[84]

Figure 2. Miscellaneous inhibitors of APN/CD13.



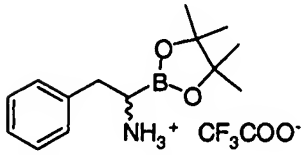
GluΨ(PO$_2$CH$_2$)Leu-Ala (6)
(mixture of four diastereoisomers)

K_i APN : 31 μM, K_i APA : 0.8nM, K_i NEP : 62 nM, K_i ACE : 53 μM[85]



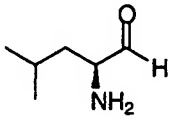
7 (racemate)

K_i APN : 2.5 μM, K_i LAP$_c$: 1 μM, K_i APB : 1.5 μM[86]



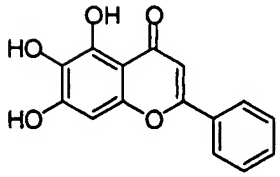
8 (racemate)

IC_{50} APN : 20 nM, IC_{50} HEDA : 50 nM[87]



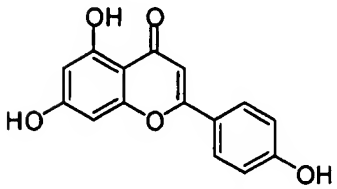
9

K_i APN : 760 nM, K_i LAP$_c$: 60 nM[88]



Baicalein (10)

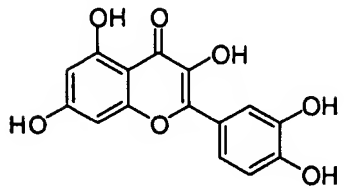
% inhibition (at maximal concentration of 300 μM)
APN : 57 %, ACE : 10 %, NEP : 36 %[90]



Apigenin (11)

% inhibition (at 100 μM) : APN : 26 %, inactive on LAP[89]
% inhibition (at maximal concentration of 300 μM) : APN : 42 %, ACE : 18 %, NEP : 31 %[90]

Figure 2. *(Continued)*



Myricetin (12)

% inhibition (at 100 μM) : APN : 25 %, inactive on LAP[89]

% inhibition (at maximal concentration of 300 μM) : APN : 48 %, ACE : 26 %, NEP : 68 %[90]

Figure 2. *(Continued)*

interesting *in vivo* antitumor potencies (30–40% inhibitory rate on S180 sarcoma) has been recently reported.[107]

3. Amastatin

(2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl-L-valine-L-valine-L-aspartic acid) (**18**) has been reported to be a slow-binding competitive inhibitor of APN.[108] It was first isolated from the culture filtrate of *Streptomyces* sp. ME98-M3.[109] and its structure has been unambiguously determined.[110] Several enantioselective syntheses of this tetrapeptide have been reported,[110,111] and some of its analogues have also been prepared in the context of a SAR study.[112]

4. Bestatin

(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine) (Ubenimex®) (**19**) is an inhibitor of various leucine and arginine aminopeptidases,[113] and an efficient inhibitor of LTA_4 hydrolase.[114–117] However, in spite of its marked toxicity and of its relative lack of selectivity toward exopeptidases, it is one of the most used compound for its APN/CD13 inhibitory effects.[118] Bestatin has been described as a slow-binding competitive inhibitor of APN,[108] and a schematic representation of **19** within the active site of APN[53,84] is depicted in Figure 3. Bestatin was first isolated from a culture filtrate of *Streptomyces olivoreticuli* (MD976-C7)[119] and its chemical structure has been subsequently ascertained.[120] Several stereoselective total syntheses of **19** have been reported,[121–130] the preparation of its stereoisomers has been performed[131] and some ubenimex derivatives or analogues such as the *para*-hydroxybestatin (**20**),[132] the 2-thiolbestatin (**21**),[133,134] the bestatin thioamide (**22**),[133,135] or the reduced bestatin **23**[136] have also been prepared.

5. Phebestin

(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-phenylalanine) (**24**) is a tripeptide produced by *Streptomyces* sp. MJ716-m3.[137] Some stereoselective syntheses of **24** have been recently reported.[125,128,129]

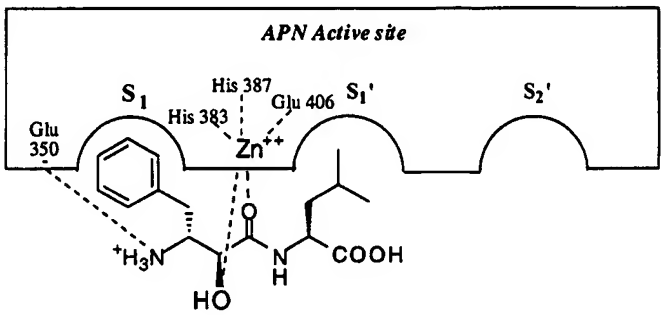


Figure 3. Binding of Bestatin to the active site of APN.

6. Probestin

(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-prolyl-L-proline) (**25**) is a tetrapeptide isolated from the culture of *Streptomyces azureus* (MH663-2F6)[138] and its structure has been unambiguously established.[139] Probestin has been described as a competitive inhibitor of APN[138] and, here also, some total syntheses have been lately described.[125,128,129]

An overview of the formulas of compounds **14–25** reveals that, except the synthetic analogue **23** prepared in its racemic form, they all possess the absolute configuration (2*S*,3*R*) which appears crucial for activity.[136] A comparable chiral framework is also existent in the side chain of the pharmacologically important series constituted by taxoids and, in this context, it is worth pointing out that numerous and various synthetic approaches to building blocks liable to lead to enantiomerically pure (2*S*,3*R*)-3-amino-2-hydroxyalkanoic structures and/or their diastereomers have attracted considerable attention.[140–185]

7. Leuhistin

(2*R*,3*S*)-3-amino-2-hydroxy-2-1*H*-(imidazol-4-ylmethyl)-5-methylhexanoic acid (**26**) has been isolated in 1991 by Takeuchi and co-workers from the culture broth of a bacteria belonging to the phylum *Firmicutes*: *Bacillus laterosporus* BM156-14F1.[186,187] This compound inhibits APN in a competitive manner with the substrate.[186] The structure of **26** and its absolute configuration have been thereafter ascertained by the same group.[188]

Several naturally occuring APN inhibitors are of vegetal origin:

8. Benzo[c]phenantridines

Benzo[c]phenantridines such as 1,2-Dimethoxy-12-methyl ^{1,3}dioxolo[4′,5′:4,5]benzo[1,2-*c*]phenanthridin-12-ium chloride or Chelerythrine (**27**) and some closely related alkaloids have recently been isolated from extracts of the Papaveraceae *Macleaya cordata* (Wild.) R. Br. Some of these compounds showed an efficacy against APN similar to that of amastatin (**18**) or bestatin (**19**). A weaker inhibitory effect on DPP-IV has also been reported.[189]

9. Curcumin

(*E*,*E*-1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) (**28**) is a yellow natural phenolic compound isolated from the rhizomes of asian perennial herbs extensively cultivated in tropical areas and belonging to the Zingiberaceae family. All these plants are of the genera *Curcuma*. The most exploited representative is *Curcuma longa* L., whose dried rhizome is the source of the spice turmeric which is widely employed in food and has a long tradition of use in folk medicine. In addition to its irreversible APN/CD13 inhibition potencies,[190] curcumin is now considered by oncologists as a potential cancer chemopreventive agent,[191,192] and clinical trials in this context are carried out in several laboratories.[193] Furthermore, curcumin possesses anti-inflammatory activity and is a potent inhibitor of reactive oxygen-generating enzymes (e.g. lipooxygenase/cyclooxygenase-2, xanthine dehydrogenase/oxidase and inducible nitric oxide synthase).[194] Curcumin hinders also the initiation of carcinogenesis by inhibiting the cytochrome P-450 enzyme activity and increasing the levels of glutathione-S-transferase. Its anti-tumor effect in the promotion and progression stages has been attributed, in part, to the arrest of cancer cells in S, G2/M cycle phase, and induction of apoptosis.[195] It has also been proposed that curcumin may suppress tumor promotion by blocking signal transduction pathways in the target cells.[196] Curcumin is a potent inhibitor of protein kinase C, EGF-receptor tyrosine kinase and I-κB kinase. In addition, curcumin inhibits the activation of NF-κB and the expression of c-jun, c-fos, c-myc.[194,197] Last, curcumin has been

proposed as a HIV-1 or HIV-2 protease inhibitor,[198] as a HIV-1 integrase inhibitor,[199] and proved to be radioprotectant.[200,201] Several chemical synthesis of **28**, involving 2,4-pentanedione and vanillin, have been reported[202–206] as well as the preparations of some of its analogues designed as angiogenesis inhibitors[207] through their ability to inhibit endothelial cell proliferation.[208]

10. Betulinic Acid

(3β-hydroxylup-20(29)-en-28-oic acid) (**29**) is a pentacyclic compound widely present in the plant kingdom. This oxidized derivative of betulin owes its trivial name to the fact that this class of lupane type triterpenes was first isolated from *Betula* ssp. (birch trees). Afterwards, betulinic acid has been obtained from various other vegetal species including *Ancistrocladus* ssp.,*Arbutus* ssp., *Diospyros* ssp., *Paeonia* ssp., *Picramnia* ssp., *Syzygium* ssp., *Tetracera* ssp., *Tryphillum* spp., *Zizyphus* ssp. One of the main current sources of betulinic acid from natural origin is the bark of plane trees (e.g. *Platanus acerifolia*) by employing a patented procedure.[209] In addition to its APN inhibitory activity in a dose-dependent manner,[210] and possibly as a partial consequence of this inhibitory potency, betulinic acid has been shown to modulate the immune response, to exhibit anti-inflammatory properties and to block HIV-1 entry into cells. It has also been reported to be a selective inhibitor of DNA polymerase β and to induce apoptosis in tumor cells. The wide range of biological properties linked to betulinic acid have recently been recapitulated and analyzed in three excellent revues.[211–213] Several hemisynthesis of **28** starting from betulin via betulonic acid[214–217] or from various naturally occuring betulinic acid derivatives such as glycosides,[218–221] sulfates,[222] or dihydroxycinnamic esters[223] have been reported.

To our knowledge, only one naturally occuring APN inhibitor originates from animal kingdom:

11. Psammaplin A

((*E*,*E*)-*N*,*N*′-Bis[(3-(3′-Bromo-4′-hydroxyphenyl)-2-oximidopropionyl]cystamine) (**30**) is a symmetrical disulfide compound bearing two hydroxyimino functional groups. This bis-bromotyrosine derivative was, almost simultaneously, first isolated in 1987 by three groups: from an unidentified marine sponge (probably of the Verongidae family) collected in Guam,[224] from a *Psammaplysilla* sp.,[225] and from *Thorectopsamma xana*.[226] Its structure has been unambiguously and independently established by these different authors. Thereafter, psammalin A has also been extracted from other sponges: *Psammaplysilla purpurea*,[225,227] *Dysidea* spp. (in this case, the authors have erroneously named «bisprasin»—the misspelled name of the psammalin A dimer—a compound which is obviously the psammalin A itself as judged by the reported formula)[228] *Aplysinella rhax*,[229–231] *Pseudoceratina purpurea*,[232] or from a two-sponge association: *Poecillastra wondoensis* and *Jaspis wondoensis*.[233,234] A biosynthetic pathway has been proposed for the formation of **30** involving modified cysteine and bromotyrosine[227,232] and, to our knowledge, only one laboratory preparation of psammalin A has been carried out starting from L-tyrosine through its its *N*,*N*′-bis-(tetrahydropyran-2-yl)oxime derivative.[235] It is also worth pointing out that a library comprising about two hundred psammalin A type derivatives has recently been prepared by Nicolaou and his co-workers by using solution phase combinatorial synthesis with the aim to evaluate their antibacterial activity.[235,236]

In addition to its very recently reported ability to inhibit APN in a non-competitive manner thus inducing a suppression of *in vitro* angiogenesis,[237] **30** has been shown to induce a variety of biological effects: (i) a significant *in vitro* antibacterial activity against *Staphylococcus aureus*[226] and methicillin-resistant *Staphylococcus aureus*[235,236,238] which is assumed to be due to its ability to inhibit DNA gyrase,[238] (ii) a cytotoxicity against various human tumor cell lines,[229,231–233] (iii) an increase in Ca^{2+} release from the heavy fraction of skeletal muscle sarcoplasmic reticulum,[228] (iv) an inhibition of topoisomerase II,[239] Leucine aminopeptidase and farnesyl protein transferase,[229], mycothiol-*S*-conjugate amidase,[240] chitinase,[231] histone deacetylase and DNA methyltransferase,[232]

and DNA replication by targeting polymerase α-primase.[241] Some antifungal and insecticidal activities have been further reported.[231]

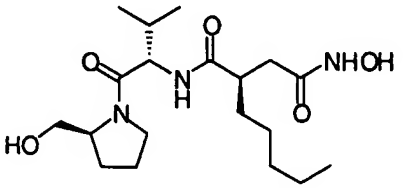
Chemical structures of APN inhibitors **13–30**, and enzyme inhibition values are depicted in Figure 4.

B. Synthetic APN/CD13 Inhibitors

Several synthetic small molecules belonging to various chemical families have been reported to inhibit APN activity.

1. α-Aminomethylketones

α-Aminomethylketones such as (*S*)-3-Amino-4-methylpentan-2-one hydrochloride (valine methyl ketone hydrochloride) (**31**)[242] have been described as potent competitive inhibitors of APN.[243]



Actinonin (13)

IC_{50} APN : 0.4 μg/mL, IC_{50} LAP : 1 μg/mL, Inactive towards APA, APB, Methionine aminopeptidase and formylmethionine aminopeptidase[94]

IC_{50} APN : 1.99 μM, IC_{50} APA : >100 μM[355]

IC_{50} APN : 0.4 μg/mL, IC_{50} APA : >100 μg/mL, IC_{50} APB : >100 μg/mL[137]

IC_{50} APN from *Bombyx mori* : 148 μM[368]

IC_{50} APN : 0.32 μg/mL[77]

IC_{50} Enkephalinaminopeptidase : 0.39 μM, IC_{50} dipeptidylaminopeptidase : 1.1μM, IC_{50} Enkephalinase A : 5.6μM[336]

91% inhibition of APN, 16.5% inhibition of APA and 13% inhibition of MDP at 100 μM, inactive towards APW[369]

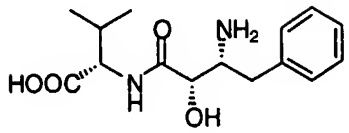
100% inhibition of APN from human seminal plasma at 100 μM[74]

IC_{50} Bacterial PDF 0.8-90 nM depending on the metal cation form of the enzyme[370]

IC_{50} Human PDF : 43 nM[358]

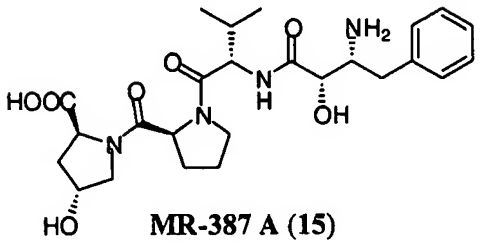
K_i Meprin α : 20 nM, K_i Meprin β : 2 μM[360]

Figure 4. Natural inhibitors of APN/CD13.



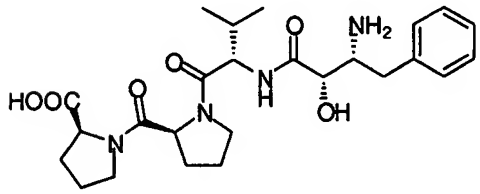
AHPA-Val (SL-387) (14)

IC_{50} APN from porcine kidney microsome : 1.2 μg/mL, IC_{50} APN from human fibrosarcoma HT1080 : 5.6 μg/mL, IC_{50} APN from human myelogenous leukemia K562 : 7.8 μg/mL[104]



MR-387 A (15)

IC_{50} APN from porcine kidney microsome : 198 nM, IC_{50} APN from human fibrosarcoma HT1080 : 218 nM, IC_{50} APN from human myelogenous leukemia K562 : 17 μM, APB from human myelogenous leukemia K562 : 651 nM[105]



MR-387 B (16)

IC_{50} APN from porcine kidney microsome : 164 nM, IC_{50} APN from human fibrosarcoma HT1080 : 201 nM, IC_{50} APN from human myelogenous leukemia K562 : 4.6 μM, APB from human myelogenous leukemia K562 : 260 nM[105]

Figure 4. *(Continued)*

2. Alkyl D-Cysteinates

Alkyl D-cysteinates display also efficient competitive APN inhibitions. Among the five esters tested, an optimal inhibitory activity has been observed with the *n*-butyl derivative (**32**).[244]

3. 3-Amino-2-Tetralone Derivatives

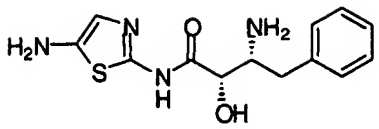
3-amino-2-tetralone derivatives such as the 2-amino-1,4-dihydro-2*H*-phenanthren-3-one hydrochloride (**33**) have been reported to be efficient and selective competitive inhibitors of APN. These compounds do not affect AP-A or AP-B and poorly inhibit LAPc.[245]

4. 3-Amino-2-Hydroxypropionaldehyde and 3-Amino-1-Hydroxypropan-2-One Derivatives

3-Amino-2-hydroxypropionaldehyde and 3-amino-1-hydroxypropan-2-one derivatives such as **34** and **35**, respectively. These competitive inhibitors of APN are very moderately active on LAPc or APB.[246]

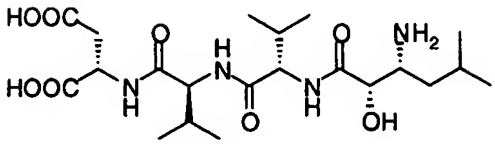
5. Flavone-8-Acetic Acid Derivatives

Flavone-8-acetic acid derivatives constitute a class of products whose the parent compound showed antiangiogenic properties.[247] In this series, products bearing a nitro group in the 2-position such as the 2′,3-dinitroflavone-8 acetic acid (**36**) proved the most potent APN inhibitors and act by reversibly binding to the catalytic site of the enzyme. These compounds present the advantage to exhibit no



(17)

Inhibits the growth of mouse S_{180} tumors by 38%[107]



Amastatin (18)

IC_{50} APN from pig kidney : 0.5 μM[371]

IC_{50} APN : 3.16 μM, IC_{50} APA : 2 μM, IC_{50} APW : 1.58 μM[355]

IC_{50} APN : 150 nM[372]

Ki APN : 50 nM, Ki LAP_c : 30 nM, Ki APA :0.15 μM [245]

IC_{50} APN : 0.58 μg/mL, IC_{50} APA : 0.54 μg/mL, IC_{50} APB : >100 μg/mL[137]

IC_{50} APN from *Bombyx mori* : 7.6 μM[368]

100% inhibition of APN, 100% inhibition of APA, 100% inhibition of APW and 18% inhibition of MDP at 100 μM[369]

100% inhibition of APN from human seminal plasma at 100 μM[74]

Ki APN : 52 nM, Ki LAP_c : 30 nM, Ki *Aeromonas* AP : 0.26 nM[357]

Ki^* APN : 20 nM, Ki^* LAP : 0.2 μM[108]

IC_{50} APM from rabbit kidney cortex : 0.4 μM[373]

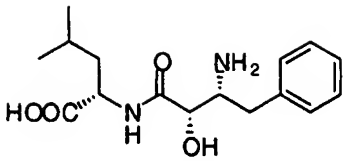
IC_{50} APM from rat blood plasma : 0.2 μM, IC_{50} APA from rat blood plasma : 8 μM [67]

IC_{50} APA : 0.54 μg/mL, IC_{50} APL : 0.5 μg/mL, IC_{50} APB : >250 μg/mL [109]

IC_{50} APA : 1.1 μM, IC_{50} LAP : 1.1 μM[112,374]

IC_{50} APW : 2 μM[375]

Figure 4. *(Continued)*



Bestatin (19)

Ki APN : 3.03 μM, Ki LAP_c : 9 nM[362]

Ki^* APN : 4.1 μM[108]

IC_{50} APN from pig kidney : 16 μM[371]

Ki APN : 1.45 μM, Ki LAP_c : 0.4 nM,

Ki *Aeromonas* AP : 18 nM[357]

Ki APN : 4.1 μM, Ki LAP_c : 20 nM,

Ki APB: 14 nM[136]

IC_{50} APM from rabbit kidney cortex : 6 μM[373]

IC_{50} APN : 89.1 μM, IC_{50} APW : 7.9 μM[355]

IC_{50} APN : 43 μM[372]

Ki APN : 3.5 μM, Ki LAP_c : 0.6 nM,

Ki APB : 6 μM[245]

Ki APN : 3.5 μM, Ki LAP_c : 0.6 nM, Ki AP *Aeromonas* P. : 20 nM, Ki APB: 6 μM[246]

IC_{50} APN : 6.2 μg/mL, IC_{50} APA : >100 μg/mL, IC_{50} APB : 0.05 μg/mL[137]

IC_{50} APN from *Bombyx mori* : 3.25 mM[368]

96% inhibition of APN from human seminal plasma at 100 μM[74]

52% inhibition of APN, 13.4% inhibition of APA, 89% inhibition of APW and 29% inhibition of MDP at 100 μM[369]

IC_{50} APN from rat blood plasma : 30 μM[67]

IC_{50} APN : 16.9 μM[210]

IC_{50} APN : 2.5 μM[190]

IC_{50} APN : 3.9 μM, IC_{50} A-LAP : 11.2 μM[249]

IC_{50} APB : 0.05 μg/mL, IC_{50} LAP : 0.01 μg/mL[119]

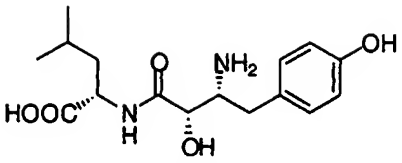
Ki APB : 60 nM, Ki LAP : 20 nM[353,356]

IC_{50} Enkephalin aminopeptidase : 1.1 μM[336]

IC_{50} APB : 0.05 μg/mL, IC_{50} LAP : 0.003 μg/mL[132]

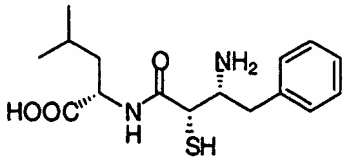
IC_{50} APW : 6 μM[375]

Figure 4. *(Continued)*



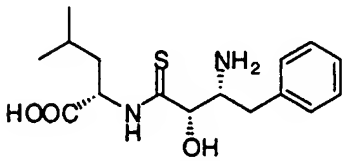
para-hydroxybestatin (20)

IC_{50} APB : 0.007 μg/mL, IC_{50} LAP : 0.02 μg/mL[132]



α-Thiolbestatin (21)

Ki APN : 4.4 μM, Ki LAP: 0.55 μM, Ki APB : 4.6 nM[133]



Bestatin thioamide (22)

Ki APN : 40.3 μM, Ki LAP: 0.33 μM, Ki APB : 2.4 μM[133]

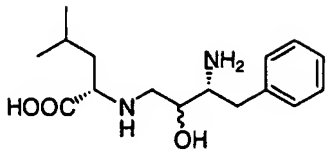
Figure 4. *(Continued)*

toxicity towards cultured human cells, to induce no apoptosis, and to be inactive on other proteases such as MMP-9, ACE, NEP, γ-glutamyl transpeptidase, cathepsin G, or DPPIV.[248]

6. N-Hydroxy-2-(naphthalene-2-ylsulfanyl)Acetamide

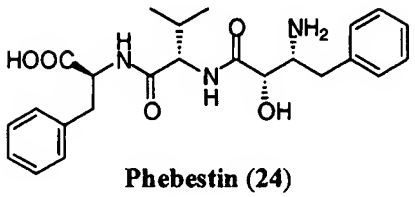
N-Hydroxy-2-(naphthalene-2-ylsulfanyl)acetamide (**37**) has recently been identified as a potent APN inhibitor. It acts in a dose-dependent manner and is inactive on metalloenzymes MMP-2, MMP-9, MMP-14, or A-LAP.[249]

The design of synthetic APN inhibitors has often been relied to structure–activity studies based on active site models derived from structural data obtained on the zinc-dependent protease thermolysin crystallized with a variety of inhibitors.[250] Molecules capable of interacting with at least the S_1 subsite of APN and which have a strong zinc-chelating group[251,252] were designed. According to these criteria, some α-aminophosphinic acids and derivatives such as **38** or **39**[253] have been prepared and proved to be very potent APN inhibitors. According to the patterns of these models, synthesis of analogs such as the iodo derivative **40** (*RB 129*) have next been performed to give rise to the radiolabelled (^{125}I)RB 129[254] which represents a useful probe to investigate the physiological role of APN.[13,255,256] In the same context, several β-aminothiols exemplified by **41**[257] or **42**[251] have been conceived and synthesized. The research in this field has then been extended to more elaborated series by Roques and co-workers, and novel sulfur-containing molecules capable of inhibiting APN such as **43**, **44**,[258] **45**, **46**[259] or **47**[253] were prepared. From these works on β-aminothiols, two products emerged: *PC 18* (*S*)(2-amino-4-methylthiobutanethiol) (**48**)[253] and *EC 27* (*S*)(2-aminopentan-1,5-dithiol) (**49**).[259] These products have essentially aroused deeper studies because they are able to induce vasopressin release by acting on the half-life of angiotensin III.[61,66,260,261]



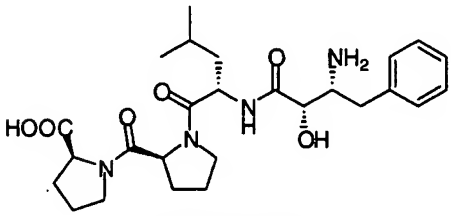
23 (racemate)

Ki APN : 2.1 mM, Ki LAP_c : > 1 mM, Ki APB: 14 nM[136]



Phebestin (24)

IC_{50} APN : 0.18 μg/mL, IC_{50} APA : 9 μg/mL, IC_{50} APB : 9 μg/mL[137]



Probestin (25)

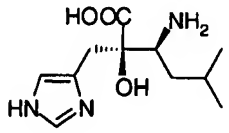
Ki APN : 19 nM[138]

IC_{50} APN : 50 nM, IC_{50} APA : 19.9 μM, IC_{50} APW : 5 μM[355]

IC_{50} APN : < 10 nM[372]

IC_{50} APN : 0.03 μg/mL, IC_{50} APA : >100 μg/mL, IC_{50} APB : 37 μg/mL[137]

IC_{50} APN from *Bombyx mori* : 74 μM[368]



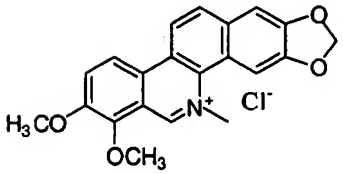
Leuhistin (26)

Ki APN : 0.23 μM[186]

IC_{50} APN : 0.2 μg/mL, IC_{50} APA : 10 μg/mL, IC_{50} APB : 13 μg/mL[137]

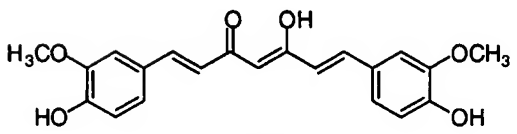
100% inhibition of APN from human seminal plasma at 100 μM[74]

IC_{50} APN from *Bombyx mori* : 0.89 mM: 089 mM[368]



Chelerythrine (27)

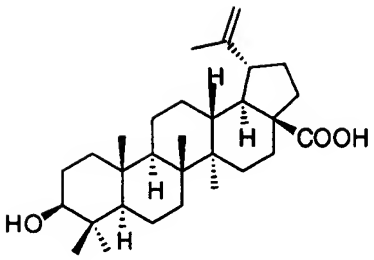
82% inhibition of APN and 38% inhibition of DPP IV at 50 μM[189]



Curcumin (28)

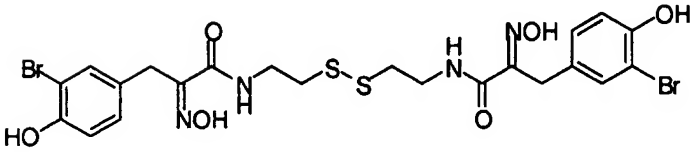
Ki APN : 11.2 μM[190]

Figure 4. (*Continued*)



IC_{50} APN : 7.3 μM[210]

Betulinic acid (29)



IC_{50} APN : 18 μM[237]

Psammaplin A (30)

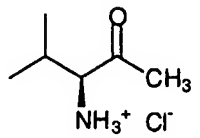
Figure 4. (*Continued*)

Formulas of the synthetic APN inhibitors **31–49**, and enzyme inhibition values are outlined in Figure 5.

C. Synthetic Dual APN/CD13 and E-24.11/CD10 (NEP) Inhibitors

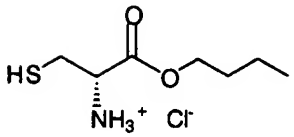
The similarities between the active sites of APN and the membrane-bound protease neutral endopeptidase 24.11 (EC3.4.24.11, CD10, NEP) led to the idea that mixed inhibitors could be developed by selecting frameworks bearing a strong zinc-chelating group and a residue able to interact with at least one subsite (S_1, S_1', and S_2') of each peptidase.[65,251,262–265] The first dual E24.11/APN inhibitors developed were hydroxamate-containing molecules such as *Kelatorphan* (**50**) or *RB 38A* (**51**)[262,266,267] whose several analogs have been synthesized and found to be also potent inhibitors of leukotriene A_4 hydrolase.[268] However, the important water solubility of these compounds is an impediment for crossing the blood–brain barrier and, consequently, for obtaining a good bioavailability. Another strategy, involving more lipophilic derivatives, led to the synthesis of *RB 101* (*N*-((*R*,*S*)-2-benzyl-3((*S*)(2-amino-4-methylthio)butyldithio)-1-oxopropyl)-L-phenylalanine benzyl ester (**52**) and *RB 120* (*N*-((*S*)-2-benzyl-3((*S*)(2-amino-4-methylthio)butyldithio)-1-oxopropyl)-L-alanine benzyl ester (**53**), two dual inhibitors in which a disulfide bridge links the APN inhibitor PC 18 with analogs (the phenylalanine analog (ST 43) in the case of **52**, or the alanine analogue in the case of **53**) of the benzyl ester of Thiorphan, a specific NEP inhibitor[269] (Fig. 6).[251,263,270] Such mixed inhibitors present the advantage to possess the above-mentioned disulfide bond which is relatively stable in plasma, in contrast to its rapid cleavage in brain, thus allowing the delivery of the NEP and APN inhibitors in their active form toward their respective target.[263] The development of such mixed inhibitors has constituted an important advance in the research of new antihypertensives and novel antinociceptive drugs devoid of opioid side effects.[264,271] (for reviews) More recently, a new generation of phosphinic acid derivatives have been prepared as NEP/APN dual inhibitors, and compounds such as **54** have been successfully tested in this context.[252,272]

Chemical structures of APN inhibitors **50–54**, and enzyme inhibition values are outlined in Figure 7.



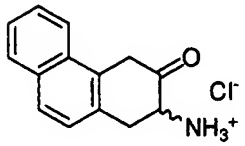
31

Ki APN : 0.55 μM[243]



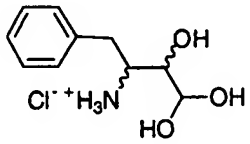
32

Ki APN : 0.18 μM[244]



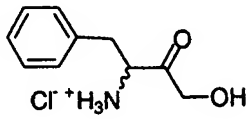
33
(absolute stereochemistry not specified)

Ki APN : 0.5 μM, Ki LAP_c : 120 μM, Ki APA: > 1 mM, Ki APB: > 1 mM[245]



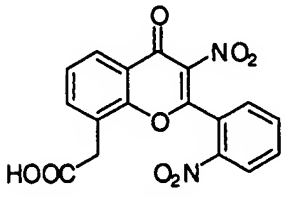
34
(absolute stereochemistry not specified)

Ki APN : 3 μM, Ki LAP_c : 0.1 mM, Ki AP *Aeromonas p.*: 30 μM[246]



35
(absolute stereochemistry not specified)

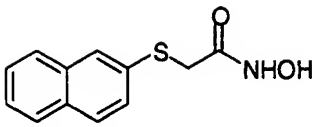
Ki APN : 1 μM[246]



36

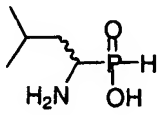
IC_{50} APN : 25 μM[248]

Figure 5. Synthetic inhibitors of APN/CD13.



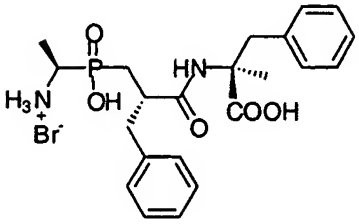
37

IC_{50} APN : 3.4 μM[249]



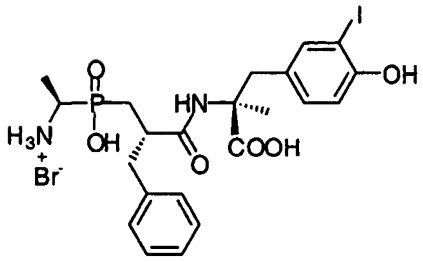
38
(mixture of R + S isomers)

Ki APN : 1.2 μM[253]



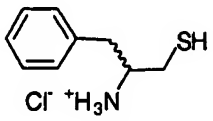
39

Ki APN : 0.6 nM, Ki APA : 0.13 μM, Ki APB : >10 μM[253]



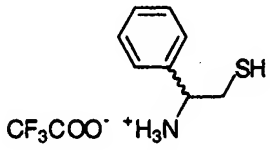
RB 129 (40)

Ki APN : 0.95 nM [254]



Phetiol (41)
(absolute stereochemistry not specified)

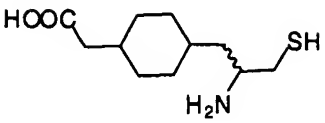
Ki APN : 5 nM, Ki PSA : 10 nM[257]



42
(absolute stereochemistry not specified)

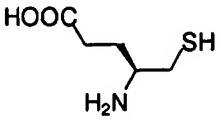
IC_{50} APN : 25 nM[251]

Figure 5. *(Continued)*



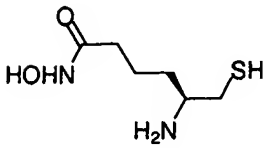
43
(mixture of R + S isomers)

Ki APN : 35 nM, Ki APA : 2.8 μM[258]



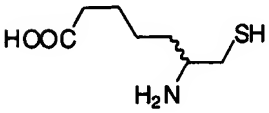
44

Ki APN : 0.12 μM, Ki APA : 0.14 μM[258]



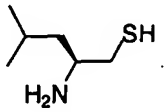
45

Ki APN : 37 nM, Ki APA : 2.0 μM[259]



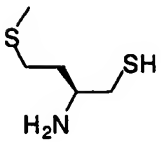
46
(mixture of R + S isomers)

Ki APN : 0.28 μM, Ki APA : 1.6 μM[259]



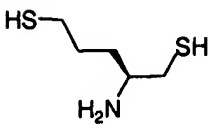
47

Ki APN : 22 nM[253]



PC 18 (48)

Ki APN : 8 nM, Ki APA : 17.2 μM, Ki APB : 1 μM[260]
Ki APN : 11 nM[339]



EC 27 (49)

Ki APN : 32 nM, Ki APA : 2.4 μM[259,261]

Figure 5. *(Continued)*

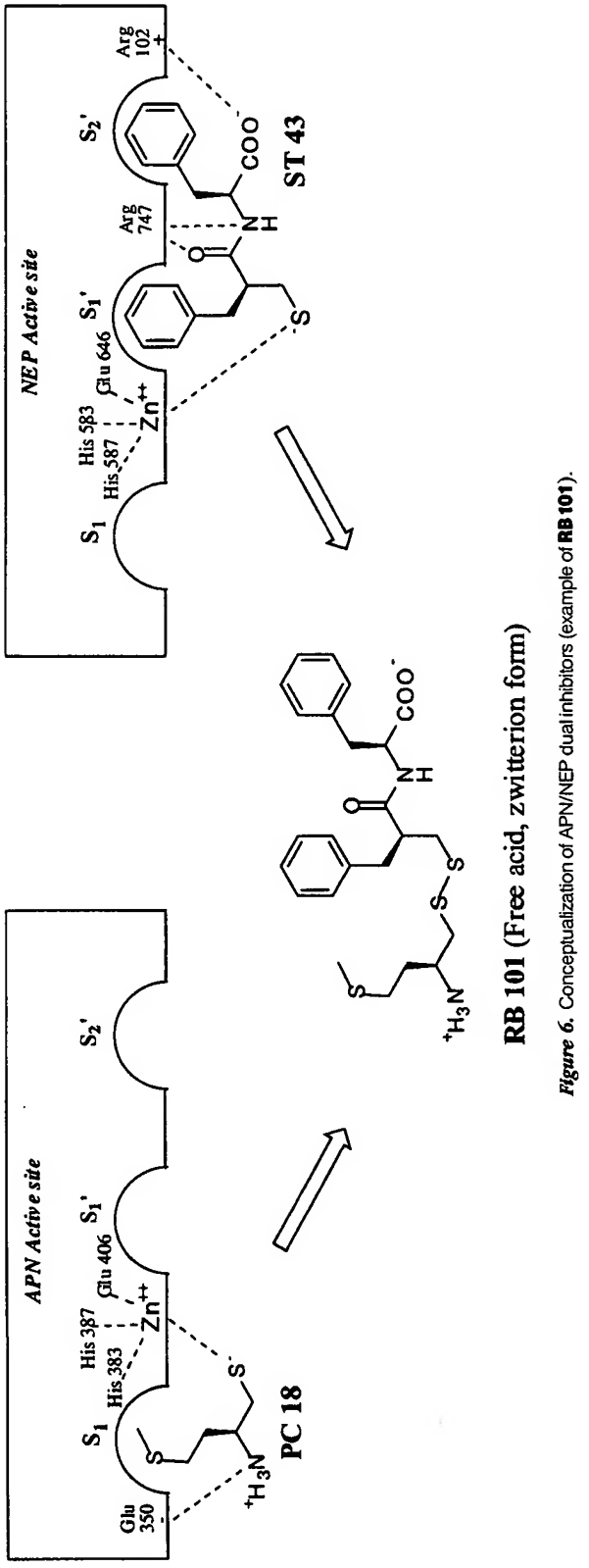
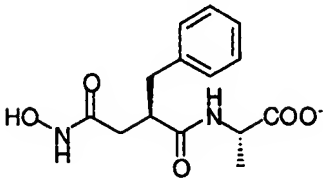
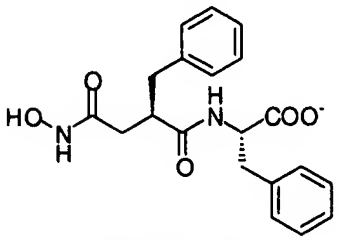


Figure 6. Conceptualization of APN/NEP dual inhibitors (example of **RB 101**).



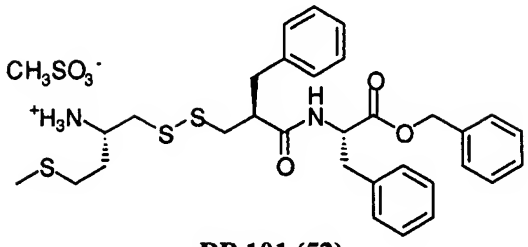
Kelatorphan (50)

Ki APN : 0.38 μM, Ki NEP : 1.8 nM[264]
Ki APN : 7 μM, Ki NEP : 1.7 nM[339]
Ki APN : 0.38 μM, Ki DAP : 0.9 nM, Ki NEP : 2.0 nM[376]
IC_{50} NEP : 46 nM, IC_{50} LTA_4 hydrolase : 5 nM, IC_{50} Aminopeptidase : 7 nM, IC_{50} ACE : >10μM[268]



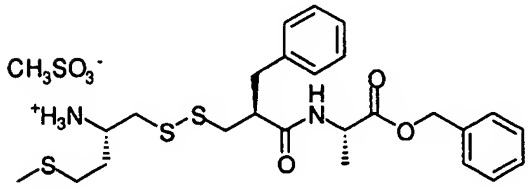
RB 38A (51)

Ki APN : 0.12 μM, Ki NEP : 0.9 nM[264]



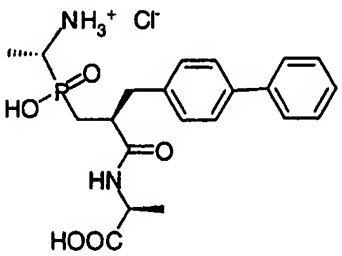
RB 101 (52)

IC_{50} APN : 16μM, IC_{50} NEP : >100μM[267]
After 15 min of preincubation with whole rat brain membrane, IC_{50} APN : 11 nM, IC_{50} NEP : 4 nM[267]



RB 120 (53)

To our knowledge, no quantitative specific value dealing with APN or NEP inhibition has been published yet.



54

Ki APN : 2.9 nM, Ki NEP : 1.2 nM[272]
Ki APN : 2.9 nM, Ki NEP : 1.2 nM, Ki ACE : 0.12 μM[252]
Ki APN : 2.9 nM, Ki NEP : 1.2 nM[339]
Ki APN : 2.9 nM, Ki APA : >1μM Ki NEP : 1.2 nM, Ki ACE : 0.12 μM[376]

Figure 7. Dual inhibitors of APN/CD13.

4. APN/CD13 INHIBITORS IN MODULATION OF CELL FUNCTIONS

The effects of some of these above described inhibitors on cell behavior have been assayed in *in vitro* approaches. Table I provides a summary of most relevant studies in the human system.

A. APN Inhibitors as Modulators of Cell Growth and Maturation

Actinonin, bestatin, probestin, and psammaplin A (at 1–100 μM concentrations) were shown to reduce the growth of human T/B lymphocytes, dendritic and cord blood $CD34^+$ cells[273–276] and human myeloid and lymphoid cell lines,[273,274,276–282] as well as the proliferation of

Table I. Effects of APN/CD13 Inhibitors in *in vitro*, in Animal and Clinical Approaches

INHIBITOR	IN VITRO	IN ANIMALS	IN CLINICAL TRIALS
Actinonin	growth ↓ apoptosis ↑ migration ↑ invasion ↓	tumor growth ↓ neovascularization ↓ pain management ↓	
Amastatin	chemotaxis ↑ angiogenesis ↓	blood pressure ↓	
Bestatin (ubenimex)	growth ↓ differentiation ↑ apoptosis ↑ migration ↑↓ invasion ↓ angiogenesis ↑↓	tumor growth ↓ fetal growth ↓ monocyte activation ↑ placental apoptosis ↑ neutrophil migration ↓ neovascularization ↓ inflammation ↓	Remission in : AML lymphoma *(monocyte & lymphocyte activation)* lung carcinoma
Betulinic acid	apoptosis ↑ angiogenesis ↓	tumor growth ↓	
Curcumin	angiogenesis ↓		
Leuhistin	invasion ↓	neutrophil migration ↓ inflammation ↓	
Probestin	growth ↓		
Psammaplin A	growth ↓ angiogenesis ↓		
PC 18 & EC 27 RB 101& RB 120		blood pressure ↓ pain management ↓	

AML, acute myeloid leukemia.

↓ Decrease ; ↑ Increase.

keratinocytes[283,284] and various tumor and endothelial cell lines.[237,285–287] A question central to APN inhibition studies is how cell growth can be turned off by APN inhibitors. APN inhibitors may alter the processing of (unknown) growth factors directly involved in the regulation of growth. In addition, several studies indicate that inhibitors like actinonin and probestin may transmit intracellular-transduction signals by interfering with the MAP kinase signaling pathway.[25,279,288,289] A second cell signaling pathway involving the Wnt-5a proto-oncogene appears also affected by inhibition of APN by actinonin.[290]

It has to be pointed out that actinonin (at a 10 μM concentration) inhibited the growth of both CD13-positive myeloid and CD13-negative lymphoma cell lines[287] suggesting that the effects induced by actinonin are not likely to be mediated by CD13. Moreover, amastatin at a concentration which inhibits APN activity was found without any effect on the growth of human myeloid cell lines[274,291].

Bestatin-mediated cell growth arrest is associated with an induction of cell maturation of clonogenic GM-CFU (granulocyte-macrophage colony forming unit) cells from human immature derived-bone marrow cells.[292,293] Similarly, treatment of human myeloid U937 and NB4 cell lines with bestatin induced phenotypic changes characteristic of macrophage (U937) or neutrophil (NB4) maturation.[280,293,294]

B. Effects of APN Inhibitors on Cell Secretion

Cell growth arrest induced by APN inhibitors correlates with alternated secretion of proinflammatory and immunosuppressive cytokines involved in pathophysiological processes. Bestatin (2.9 μM) increased the levels of IL-8 secreted by endothelial cells,[295] and of IL-1 release from mouse peritoneal macrophages and IL-2 release from concanavalin-stimulated T cells.[296] Probestin induces the synthesis and release of TGF-β1.[41,297]

C. Effects of APN Inhibitors on Apoptosis

Recent observations point to the involvement of APN in the process of apoptosis (programmed cell death). Bestatin and actinonin (starting 30 μM) induce apoptosis in a large variety of cell lines, i.e. myeloid (P39/TSU, HL-60, U937, NB4) and lymphoid (Jurkat, BJAB, NALM6, BOE) cells, and carcinoma (fibrosarcoma, cervical, and lung carcinoma).[274,282,287,291,298,299] Betulinic acid induces apoptosis in the HT29 colon cancer cell line (26 μM)[84] and in acute leukemia cells (50 μM).[300]

D. Effects of APN Inhibitors on Cell Motility

In a general way, cell motility (migration and invasion) may be influenced by the processing of chemokines and/or degradation of the extracellular matrix (ECM). The two small proteins with chemotactic activity, MCP-1 and f-MLP, are *in vitro* hydrolyzed by APN/CD13. With regard to MCP-1, there is no current data reporting the potential action of APN inhibitors on the MCP-1-mediated migration. Actinonin and amastatin were able to enhance the chemotactic response of human neutrophils toward f-MLP.[301] One explanation of the effects of actinonin or amastatin would be that both inhibitors prevent the inactivation of f-MLP by APN, to further enhance the f-MLP-mediated chemotactic response. It has however to underline that both inhibitors weakly inhibited APN enzymatic activity over the range from 10^{-8} to 10^{-4} M, concentrations that are effective on neutrophil migration.[301]

APN inhibition by actinonin or bestatin significantly enhanced the *in vitro* migration of eosinophils across HUVEC monolayers.[302] Moreover, actinonin, bestatin as well as leuhistin (50–150 μM) significantly blocked the invasion of various human metastatic tumor cells into reconstituted basement membranes[303,304] or into Matrigel.[21,305–307] These latter data suggested that

APN could be indirectly involved in type IV collagen degradation by activating type IV procollagenase/proMMP-9.[17,303,304] Recent studies demonstrated that soluble APN/CD13 induces *in vitro* chemotactic migration of T lymphocytes, and that bestatin at high concentration (580 μM) abolishes this process, suggesting that the enzymatic activity of APN was responsible for the chemotactic activity.[34,36,304,308]

5. APN INHIBITORS AND ANGIOGENESIS

The demonstration of the participation of APN in angiogenesis has come from recent studies in which blocking APN activity by APN inhibitors resulted in the perturbation of "angiogenic" assays (Table I).

A. In Vitro Assays

APN/CD13 is expressed on the human umbilical vein endothelial cells (HUVECs) of angiogenic, but not normal, vasculature.[309] Bestatin, betulinic acid, amastatin, curcumin, and psammaplin A (10–250 μM) abrogate the ability of the HUVECs cultured on matrigel to organize a capillary network[20,190,237,310–312] without altering their proliferation rates.[310] In contrast, one study underlines the proangiogenic effect of bestatin (8–250 μM) which instead causes matrix degradation and stimulates the invasion of microvascular endothelial cells into a fibrin matrix.[313]

B. In Vivo Assays

In the chorioallantoic membrane (CAM) assay, the angiogenic response is determined by measuring the number of avian extraembryonic capillary vessels that grow within a matrix polymer (containing an angiogenic molecule such as fibroblast growth factor-2/FGF-2) placed on the yolk sac membrane of a 4 day embryo in culture.[314] The chick vasculature expresses a phenotype APN/CD13, and subsequent treatment with bestatin or actinonin (200 μg) inhibited FGF-2-induced angiogenesis.[309] In the mouse retinal neovascularization model, bestatin (200 μg/mouse) leads to the blockade of hypoxia-induced retinal neovascularization in mice.[309] The intraperitoneal administration of bestatin (50–100 mg/kg/day) after the orthotopic implantation of B16-BL6 melanoma cells into mice reduces the number of vessels oriented toward the established primary tumor mass on the dorsal side of mice.[311]

6. EFFECTS OF APN/CD13 INHIBITORS IN ANIMAL MODELS

Compiled data documenting the involvement of APN/CD13 in pathophysiological events (cancer, inflammation, infection, pain suppression) have come from studies which blocked APN activity in rodent models (Table I).

Studies in rats indicate that administration of bestatin leads to the inhibition of fetal growth and the induction of placental apoptosis.[315,316] The *in vivo* anti-cancer activities of bestatin and betulinic acid have been reported through their capacities to inhibit the growth of syngeneic tumor (leukemia/melanoma/ovarian/hepatoma/gastric carcinoma) cells implanted in mice[16,213,309,310,317–325] and rats.[319,326,327] Doses as low as 0.5 mg/kg for bestatin and 5 mg/kg for betulinic acid were used in these studies. Moreover, high doses (up to 500 mg/kg) did not lead to any cytotoxic effect in mice.

Bestatin, leuhistin, and betulinic acid have been investigated for anti-inflammatory properties. Betulinic acid possessed moderate ant-inflammatory abilities at relatively high concentrations

(100 mg/kg/mouse, i.v.).[213] In contrast, bestatin and leuhistin inhibit acute inflammation associated the accumulation of polymorphonuclear neutrophils in a mouse model (2 mg/kg, i.v.).[57,328] Moreover, oral administration of bestatin (5 mg/kg) in carcinoma-bearing mice induces generation of cytotoxic T cells and NK (natural killer) cells.[317]

Angiotensins II and III are two peptide effectors of the brain rennin–angiotensin system that participate in the control of blood pressure, increase water consumption and vasopressin release. In hypertensive rats, infusion of amastatin (16 nmol/min i.v.) prevents degradation of angiotensins associated with blood pressure decrease.[67,329]. In the mouse brain, APN inhibition by PC18 or EC27 (10–300 μg injected intracerebroventricularly) increases the half life of angiotensin III, resulting in enhanced vasopressin release.[61,66,260]

Several studies report that bestatin exerts anti-infectious properties by augmenting host resistance to bacterial, viral or fungal experimental infections in mice by inducing neutrophil and macrophage activation[330,331] and enhancing antibody production.[330–335]

Finally, in the central nervous system, enkephalins which modulate responses to painful stimuli, are inactivated by APN and the membrane-bound protease neutral endopeptidase 24.11 (EC3.4.24.11, CD10). This led to the idea that inhibition of these enzymes (alone or in combination) could achieve clinically efficient analgesia. Actinonin as well as the dual inhibitors RB101 and RB120 (9 mg/kg, i.v.; 80 mg/kg, i.p.) exhibited analgesic properties against chronic pain in rats and mice.[261,263,267,336,341]

7. EFFECTS OF BESTATIN IN CLINICAL TRIALS

In first clinical trials, bestatin (30 mg/daily) has been used to treat patients with acute and chronic myeloid leukemias (AML, CML) and lymphomas.[342–346] Therapeutic efficacy was demonstrated by a prolongation of survival in patients with AML[345,346] and lymphomas,[342,343,347] and in promoting graft versus leukemia effects in patients following allogeneic bone marrow transplant.[348]

In a phase Ib trial, activation of blood monocytes and increase in the CD4/CD8 lymphocyte ratio were observed in Hodgkin's and non-Hodgkin's lymphoma patients treated orally with high doses of bestatin (90–180 mg/daily/60 days) following autologous bone marrow transplantation.[330,334,349]

In phase III trials in resected stage I squamous cell lung carcinoma, survival was statistically better for patients who were treated with bestatin (30 mg/daily/2 years) as a post-operative adjuvant therapy than those who received a placebo.[350,351]

8. CONCLUDING COMMENTS AND PERSPECTIVES

APN/CD13, is useful in defining clinical subgroups of patients with various malignancies or inflammatory diseases. The use of natural and synthetic APN inhibitors has revealed that APN/CD13 participates to the control of major biological processes such as proliferation, secretion and apoptosis. Dysregulation of APN/CD13 in tumors is often linked to tumor invasion and angiogenesis. Studies on non-hematopoietic cells suggest that APN/CD13 may influence cell migration and invasion. APN/CD13 inhibitors have been shown to alter angiogenesis in *in vitro* and *in vivo* assays. Documented evidence underlines both the antiangiogenic and proangiogenic effects of bestatin.[309,310,313] Figure 8 summarizes our current understanding of the involvement of APN inhibitors in the modulation of these events. The detailed molecular mechanisms underlying these effects are however yet unclear.

Importantly, the requirement for APN in these processes has been mostly confirmed with studies in which APN/CD13 expression was blocked by neutralizing CD13 antibodies[20,285,303,309,310] or antisense CD13 oligonucleotides,[20,41,352] or enhanced by the use of CD13 transfectants.[17]

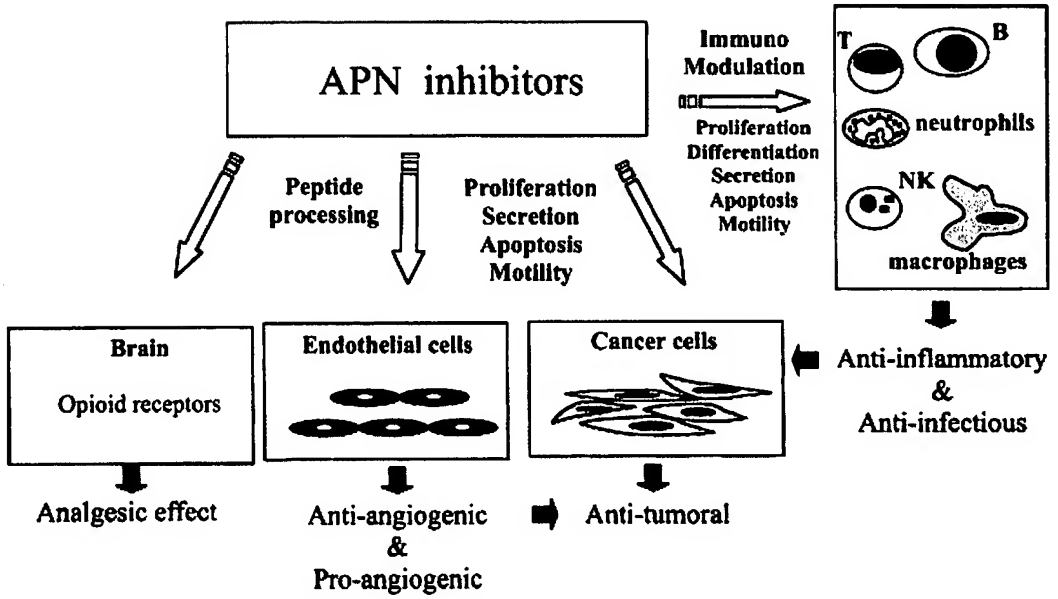


Figure 8. Biological effects of APN/CD13 inhibitors. The actions of APN inhibitors in vitro and in vivo (animal models) are diverse; they may directly target cancer cells, or act indirectly against targets by activation of immune cells (T, B, neutrophils, natural killer/NK cells, macrophages) or alteration of angiogenesis (endothelial cells). In the brain, APN inhibitors exhibit analgesic properties by increasing the levels of Met-enkephalin.

It has however to be pointed out that most of APN inhibitors lack tight specificity by inhibiting other membrane-bound metalloproteases or secreted matrix metalloproteinases (MMPs) (Table I). For example, bestatin interacts with leucyl-aminopeptidase (EC3.4.11.1, oxytocinase, Leu-AP), aminopeptidase B (EC 3.4.11.6, AP-B) and aminopeptidase W (EC 3.4.11.16, AP-W)[136,353–357] thus suggesting that some of the observed chemotherapeutic actions of bestatin may be due to inhibition of other cell surface peptidases. Actinonin was recently shown to interact with human peptide deformylase,[358,359] meprin α (EC 3.4.24.18, endopeptidase 24.18),[360] and MMP-2.[361] Amastatin and probestin in the low micromolar range (1.5–20 μM) inhibit aminopeptidase A (EC 3.4.11.2, AP-A) and AP-W.[109,355,362] Leuhistin inhibits AP-A and AP-B to the same degree than APN.[186] Curcumin and betulinic acid block MMP-9 expression and collagenase activity through inhibition of NF-κB activation.[363–367] In addition, the use of available APN inhibitors in some experimental situations has revealed complex effects on cell behavior. As mentioned in paragraph 4.A, CD13-positive and CD13-negative cell lines are equally sensitive to the growth-inhibitory effect of actinonin (50–260 μM)[287] thus emphasizing that actinonin may induce unspecific cytotoxic side-effects. Moreover, betulinic acid inhibits tube formation of bovine aortic endothelial cells at a concentration which had no effect on the cell viability and *in vivo* APN activity of endothelial cells, thus indicating an APN-independent mode of action of betulinic acid.[312]

Together, these observations emphasize the need for more specific and targeted APN inhibitors to (re)evaluate the actions of APN/CD13 in pathophysiological processes. Future consideration has to be given to the obtention of the three-dimensional structure of APN determined by NMR spectroscopy to help APN inhibitor design strategy. Further *in vitro* and *in vivo* studies with promising non cytototoxic APN inhibitors (such as psammaplin A, phosphonic derivatives, flavone-8-acetic acid derivatives) are also required before clinically prescribing an APN inhibitor as an anti-cancer or anti-inflammatory agent.

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MEMBRANE BOUND MEMBERS OF THE M1 FAMILY: MORE THAN AMINOPEPTIDASES

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Abstract: In mammals the M1 aminopeptidase family consists of nine different proteins, five of which are integral membrane proteins. The aminopeptidases are defined by two motifs in the catalytic domain; a zinc binding motif HEXXH-(X_{18})-E and an exopeptidase motif GXMEN. Aminopeptidases of this family are able to cleave a broad range of peptides down to only to a single peptide. This ability to either generate or degrade active peptide hormones is the focus of this review. In addition to their capacity to degrade a range of peptides a number of these aminopeptidases have novel functions that impact on cell signalling and will be discussed.

Keywords: aminopeptidase, APA, APN, TRH-DE, ERAAP, Angiotensin IV, IRAP.

INTRODUCTION

The superfamily of zinc-dependent metalloproteases share a common pattern of primary structure [1-3], and its members are involved in many crucial aspects of development and cellular physiology. Depending on the topology of their catalytic sites, these enzymes have been classified into different categories [4]. One category, the gluzincins is characterised by the HEXXH motif, containing two zinc-coordinating Histidine residues, and a Glutamate residue as the third zinc ligand [4]. Under this category, enzymes are further classified into a number of distinct families [5], including the M1 family (M stands for metallo-type) of zinc-dependent aminopeptidases.

Aminopeptidases catalyse the cleavage of amino acids from the N-terminus of protein or peptide substrates. These enzymes play critical roles in many physiological processes such as protein maturation and regulation of peptide hormone levels. They are widely distributed in animal tissues and found in many subcellular organelles, in the cytoplasm, and as integral membrane proteins [4, 6, 7]. The M1 family is defined by two conserved sequence elements in the catalytic domain; the HEXXH-(X_{18})-E consensus zinc-binding motif and the GXMEN exopeptidase motif. In humans, the family consists of nine enzymes; five

transmembrane proteins: aminopeptidase A (APA) [8], thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE)[9], aminopeptidase N (APN) [4, 5], adipocyte-derived leucine aminopeptidase (A-LAP) [10] and insulin-regulated aminopeptidase (IRAP) [11, 12]; and four non transmembrane enzymes: aminopeptidase B (APB) [13, 14], puromycin-sensitive aminopeptidase (PSA) [15], leukotriene A4 hydrolase (LTA4H) [16-19] and the recently identified leukocyte-derived arginine aminopeptidase (L-RAP).

This family of enzyme exhibits differing degrees of substrate selectivity, with IRAP, APA and APN, capable of cleaving a range of neuropeptides, to TRH-DE, which only cleaves one peptide, TRH. The physiological roles/significance of the M1 family of aminopeptidase is dictated not only by their substrate selectivity but is also dependent on their tissue specific expression. In addition, the activity of the aminopeptidases with broad substrate specificity may also be regulated by their distribution in the cell; their distribution within intracellular compartments may restrict exposure of their catalytic domain to peptide substrates in contrast to their presence on the plasma membrane.

M1 aminopeptidases are indirectly involved in cellular signalling by their roles in either the degradation or the generation of biologically active peptides. What is interesting about this class of aminopeptidases is that the *in vivo* substrates are not in all cases clearly defined and for some enzymes remain unknown. This lack of information can be and is overcome by the development of specific inhibitors. Another interesting characteristic of this family of aminopeptidases is the bifunctional nature of a few members that may potentially impact on the cellular signalling events modulated by these enzymes. This is exemplified by LTA4H that catalyses both the activation of the inflammatory mediator leukotriene B4 and cleavage of specific peptides with N-terminal arginine (although the *in vivo* peptide substrates remain to be elucidated). The range of functions of M1 aminopeptidases is reflected in the different names given to the same protein, with nomenclature determined upon identification of a particular functional property of the protein.

In this review, the five membrane bound members of the M1 aminopeptidase family (Table 1) are discussed with a focus on IRAP and examples are given of multi-functional roles, usefulness of specific inhibitors for the elucidation of the physiological actions of these aminopeptidases and signalling events associated with them.

Table 1. Summary of the membrane bound M1family aminopeptidases

Transmembrane M1 Enzyme	EC	Substrate Examples
Aminopeptidase A (APA)	3.4.11.7	Ang II, CCK-8
Thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE)	3.4.19.6	TRH
Aminopeptidase N (APN)	3.4.11.2	Ang III, enkephalin
Adipocyte-derived leucine aminopeptidase (A-LAP)	3.4.11.-	Ang II, Ang III, kallidin
Insulin-regulated aminopeptidase (IRAP)	3.4.11.3	Vasopressin, Oxytocin, enkephalin

Aminopeptidase A (APA); angiotensinase; BP1/6C3; glutamyl aminopeptidase, EC 3.4.11.7.

Aminopeptidase A is an ectoenzyme that selectively hydrolyses acidic residues from the amino terminus of peptides, including the vasoactive Ang II [20] and the centrally active chloecystokinin-8

(CCK-8) [21]. It catalyses the removal of N-terminal aspartate residue of Ang II to Ang III, a degradation step that is important in local regulation of blood pressure in mammals. This reaction is potentially bi-functional: in the brain it may be responsible for the production of the active neuropeptide Ang III whereas in the periphery it may be the first metabolic step for the degradation of Ang II.

Elegant work by Llorens Cortes and others to delineate the physiological roles of ANG III in the central nervous system have utilized the specific inhibitors EC33 and EC27 (or PC18) to APA and APN respectively [20, 22]. Studies using these inhibitors suggest Ang III may be the active ligand that binds to, and activates, the brain angiotensin AT1 receptor in preference to Ang II. This is despite Ang II and Ang III having similar affinities for the AT1 receptor and half life studies by Harding *et al.* demonstrating that Ang III is metabolized significantly more rapidly than Ang II in the brain [23, 24]. Vasopressin is generated in the magnocellular neurones of the supraoptic (SON) and the paraventricular nuclei (PVN) and released in the posterior pituitary. This release is stimulated by angiotensin II and III and blocked by the angiotensin AT_1 receptor antagonists. Inhibition of APA by EC33 inhibits Ang II-induced vasopressin release in a dose dependent manner suggesting that the conversion of Ang II to Ang III is essential for this process [20]. In addition, injection of the APN inhibitor alone results in an increase in vasopressin release [20]. Since Ang III is a substrate of APN, administration of the APN inhibitor could result in increased levels of the neuropeptide leading to activation of the AT1 receptors and subsequent increased vasopressin release. A controversial issue with the hypothesis that Ang III is the centrally active peptide acting on the AT1 receptor is the absence of the receptors in the magnocellular neurons of the SON and PVN leading to speculation of an indirect effect or even a novel Ang III receptor [25].

In addition a number of other studies have investigated the potential significance of Ang III as the active neuropeptide in the central control of blood pressure. Recently Harding et al investigated the effect of central infusions of Ang II, Ang III and stable analogues (d-Asp(1)Ang II and d-Arg(1)-AngIII) with and without the APA and APN inhibitors on elevations in mean arterial pressure (MAP) [26]. Pretreatment with EC33 blocked the blood pressure activity of central infusion of d-Asp(1)Ang II but had no effect on increases in MAP elicited by central d-Arg(1)-AngIII infusion. The specific AT_1 receptor antagonist , losartin, blocked all of the blood pressure responses described indicating that the effects were mediated by the AT_1 receptor. Therefore these studies also support the concept of Ang III as an important, centrally active, angiotensin ligand [26].

The peripheral tissue distribution of APA determined by *in situ* hybridisation and immunohistochemistry is identical to those areas in which Ang II has physiological effects [27, 28, 29]. Furthermore, the highest APA mRNA and activity levels occur in the kidney and ileum, which are the principal tissues for Ang II and CCK-8 synthesis. It may indicate a regulatory role of APA in Ang II and CCK-8 metabolism [28]. In contrast APA activity and expression in spontaneously hypertensive rats (SHR) are significantly elevated in the kidney, with modest increases in brain, heart and adrenal gland. This elevation in APA levels in the kidney was reduced by the treatment with the ACE inhibitor captopril along with reduced blood pressure in SHR suggesting APA levels are in part regulated by the renin-angiotensin system [30].

Thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE; EC 3.4.19.6)

Thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE: EC 3.4.19.6), formerly known as thyroliberinase, TRH-specific aminopeptidase, and pyroglutamyl-peptidase II or pyroglutamyl-aminopeptidase II (PAP II), is also a member of the M1 family of Zn-dependent aminopeptidases [9, 19, 31]. TRH-DE is located on the plamsa membrane of cells and a soluble form is present in serum, both degrade TRH rapidly. Previous biochemical studies have indicated that the membrane-bound and serum TRH-DE are derived from the same gene, whereby the serum enzyme is generated by proteolytic cleavage of the particulate liver enzyme [32]. The highest level of activity of the membrane-bound TRH-DE is found in brain and significant activities are also detected in retina, lung and liver [33].

TRH-DE is a unique member of the M1 family of aminopeptidases as it specifically catalyses the degradation of thyrotropin-releasing hormone (TRH; pyroglutamyl-L-histidyl-prolylamide-NH_2) by cleavage of the L-pyroglutamyl/histidinyl bond [34-38]. TRH stimulates the release of thyrotropin and prolactin from the anterior pituitary and potentially functions as a neurotransmitter within both the central and peripheral nervous systems. TRH-DE does not cleave any of the other known pyroGlu-containing neuropeptides (eg lutenizing hormone releasing hormone, neurotensin) [39-41] and is therefore an example of a neuropeptide-specific peptidase. Given the unique nature of TRH-DE the development of specific inhibitors would be invaluable to further investigate the functions of TRH and TRH-DE in the central nervous system and potentially as a therapeutic drug to enhance TRH actions.

Aminopeptidase N (APN; CD13; Alanyl aminopeptidase; EC 3.4.11.2)

APN is a type II membrane-bound protein consists of 967 amino acids, with a small N-terminal cytoplasmic domain, a single transmembrane domain and a large extracellular ectodomain that contains the active site [42]. Sequence comparison demonstrates that APN is identical to CD13 antigen [43] which was originally identified on subsets of normal and malignant human myeloid cells and highly expressed in human monocytes. Abnormal expression of APN was also observed on malignant lymphocytes. APN/CD13 is thought to play a role in the modulation of proliferation and function of various immune cells in humans, and this idea has been supported by accumulating evidence [44-49].

APN degrades regulatory peptides by removing the N-terminal, preferentially, neutral residues in diverse cell types, including small intestinal, renal tubular and lung epithelial cells, macrophages, granulocytes, and synaptic membranes of neurons [42, 50-55]. *In vitro* studies on the APN-mediated metabolism of vasoactive peptides indicates a significant role of APN in modulating their levels in the circulation in vivo [56-58]. Furthermore, APN also participates in the metabolism of renin-angiotensin system by cleaving Ang III to Ang IV [20, 59] (described above) as well as the metabolism of enkephalin [60] in the brain.

In addition to its role as an aminopeptidase, APN has also been shown to be a receptor of human [61] and porcine coronaviruses [62], a human herpesvirus [54, 63] and *Bacillus thuringiensis* CrylA(c) toxin [64] in their target tissues. However, the virus- and toxin-receptor interaction sites seems to be distinct from the enzymatic site and aminopeptidase activity is not necessary for viral infection or toxin activity [54, 64, 65].

Adipocyte-Derived Leucine Aminopeptidase (A-LAP); endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP); aminopeptidase regulator of TNFR1* shedding (ARTs-1) EC 3.4.11.

A-LAP was originally identified as an aminopeptidase from adipocytes displaying aminopeptidase activity with specificity to Leu, followed by Met, Cys and Phe. The natural substrates of A-LAP include a number of bioactive neuronal peptides including angiotensins (Ang II and Ang III) and kallidin, indicating a potential functional role in the brain and in the regulation of blood flow [66]. In addition the localisation of A-LAP in rodent kidney where Ang II and bradykinin are produced, support a role in regulating blood pressure [66].

A-LAP was also identified by Cui *et al.* as the protein whose binding to the extracellular domain of type 1 tumour necrosis factor receptor I(TNFR1) increases TNFR1 shedding and results in decreased membrane-associated TNFR1 in both human pulmonary epithelial and umbilical vein endothelial cells. However, A-LAP itself does not possess TNFR1 sheddase activity, but regulates the cleavage and was therefore named aminopeptidase regulator of proteolytic cleavage of TNFR1 shedding (ARTS-1) [67]. Confocal microscopy studies further confirmed the colocalisation of ARTS-1 on the apical cell membrane of ciliated human bronchial epithelial cells with TNFR-1 [67]. In addition the same group has also demonstrated that ARTS-1 regulates the shedding of the interleukin-6-receptor (IL-6Rα) [68]. This was demonstrated by identifying a direct interaction between ARTS-1 and IL-6Rα, increased IL-6Rα shedding with increased levels of ARTS-1 and the absence of constitutive shedding of IL-6Rα in *arts-1* knock-out cells. The ability of ARTS-1 to promote the shedding of these two cytokine receptors suggests a role for ARTS-1 in the modulation of inflammatory proceedings.

A third group recently identified A-LAP as the aminopeptidase that is required for the trimming of intracellular peptides in the endoplasmic reticulum (ER) to precise lengths for the binding to major histocompatibility complex (MHC) class I molecules [69]. Hence the third name Endoplasmic Reticulum Aminopeptidase associated with Antigen Processing (ERAAP) [69]. MHC class I molecules present ERAAP derived antigenic peptides from cellular proteins to the cell surface enabling T cells, carrying the CD8 antigen, to detect tumours and cells harbouring foreign pathogens. A-LAP/ERAAP mRNA has been detected in all tissues analysed in keeping with the presence of MHC class I molecules on nearly all cells of the body, including neurons [66, 70].

IRAP (cysteine aminopeptidase, oxytocinase, placental leucine aminopeptidase (P-LAP); EC 3.4.11.3)

Insulin-regulated aminopeptidase, IRAP, was initially cloned from a rat epididymal fat pad cDNA library as a marker protein (vp165) for a specialized vesicle containing the insulin-responsive glucose transporter, GLUT4 [11]. The same protein was cloned concurrently from human placental cDNA library as oxytocinase [12], an enzyme which is thought to have an important role in degrading oxytocin during pregnancy. More recently, IRAP was purified and isolated from bovine adrenal membranes as the specific binding site for angiotensin IV, known as the AT_4 receptor [71]. Although isolated by three independent groups from different tissue sources and involved in distinct physiological roles, several properties and characteristics of this protein remain consistent.

IRAP is a type II membrane-spanning protein such that when at the plasma membrane the catalytic site is extracellular [11]. The N-terminal cysteine residues contained in vasopressin and oxytocin peptides are the preferential targets for the enzyme although a number of peptides that do not contain cysteine residues are also hydrolysed by IRAP *in vitro*. These include Lys-bradykinin, Ang III, met-enkephalin, dynorphin A, neurokinin A and neuromedin B [72, 73, 74].

In insulin-responsive cells in the basal state, a major proportion of IRAP and GLUT4 are co-localised inside the cell in specialised post-endosomal vesicles and in the trans-golgi network. Both GLUT4 and IRAP rapidly translocate to the plasma membrane under insulin stimulation where GLUT4 mediates insulin-stimulated glucose uptake (reviewed in [59]). However the physiological relevance of the translocation of IRAP to the cell surface in response to insulin in adipocytes and skeletal muscle remains to be elucidated.

IRAP is the only transmembrane enzyme of the M1 family of aminopeptidases containing a relatively large intracellular domain (109 amino acids). In GLUT4 vesicles, the amino-terminal end of IRAP projects into the cytoplasm and the large catalytic domain is present within the lumen of the vesicle. At the plasma membrane the catalytic domain is extra-cellular and the amino terminal domain cytoplasmic. The studies on the cytoplasmic tail of IRAP have demonstrated an involvement in the retention of GLUT4-containing vesicles in insulin responsive cells.

The amino terminus of IRAP contains two dileucine motifs and two acidic regions, which are associated with protein trafficking [75]. Microinjection of the cytoplasmic domain of IRAP into 3T3-L1 adipocytes results in rapid translocation of GLUT4 to the plasma membrane in an insulin independent manner. A peptide consisting of residues 55-82 of the amino terminus, containing one of the dileucine motifs and acidic clusters, was sufficient to cause GLUT4 translocation [76]. Three proteins have been identified to date that interact with the IRAP cytoplasmic tail. The poly(ADP-ribose) polymerase Tankyrase was interacts with amino acids 96-101 of IRAP (RQSPDG) just proximal to the transmembrane domain; subsequently a tankyrase-binding motif has been identified (RXXPDG) [77, 78]. Although tankyrase is associated with the golgi, it does not appear to be involved in the retention of GLUT4 vesicles [77]. In contrast acyl-coenzyme A dehydrogenases (ACDs), may be involved in retention of GLUT4-vesicles retention [79]. Inhibitors of ACDs were demonstrated to cause separation of ACDs from IRAP *in vitro* and also the translocation of GLUT4 to the plasma membrane in permeabilized 3T3-L1 adipocytes [79]. FHOS, the formin homolog that is overexpressed in spleen, was isolated from human skeletal muscle library using IRAP 55-82 as bait in a yeast two hybrid screen [80]. In addition to its interaction with IRAP, FHOS, and a splice variant were found to bind the actin binding protein, profilin IIa [80]. Overexpression of FHOS or FHOS78 in the L6 cell line resulted in enhanced basal and insulin-induced glucose uptake although this was not the case in 3T3-L1 differentiated adipocytes [80]. Tojo *et al.* speculate that FHOS and its splice variant, FHOS78, may play a role in tethering GLUT4 vesicles to the cytoskeleton through their interaction with profillin IIa.

IRAP (P-LAP) is also produced by the placenta as a membrane-bound protein and then secreted into maternal serum [81]. The soluble form of P-LAP is derived from the membrane-bound enzyme by post-translational proteolytic cleavage near the transmembrane domain by a specific secretase [12]. In spite of

the widespread tissue distribution of the membrane-bound P-LAP/IRAP, the soluble form of P-LAP is only detected in serum during pregnancy with levels increasing throughout gestation [12, 82]. Peptide hormones such as oxytocin, vasopressin and Ang III can act either as uterine contractors or as vasoconstrictors and are all degraded by P-LAP (*in vitro*) [73], suggesting a regulatory effect of P-LAP/IRAP on uterine tonus and uteroplacental blood flow [83]. Moreover, abnormally high levels of serum P-LAP/IRAP have been observed in pregnancy-associated transient diabetes insipidus characterised by polyuria that is induced by increasing clearance rate of vasopressin [84-86]. In contrast the activity of IRAP in severe pre-eclampsia serum was lower than that in normal pregnancy [83]. These findings suggest that P-LAP/IRAP may play a critical role in the maintenance of normal pregnancy.

A potential role for IRAP has been suggested as part of a negative feed back loop for the regulation of both oxytocin and vasopressin. In a study on human vascular endothelial cells, activation of the oxytocin receptor by oxytocin, (a substrate for IRAP) was found to translocate IRAP to the cell surface [87]. Nakamura et al demonstrate that activation of a protein kinase c-dependent pathway by oxytocin acting on the oxytocin receptor triggers the translocation of IRAP (oxytocinase) to the plasma membrane. Potentially this would result in the degradation and therefore inactivation of oxytocin. More recently a similar scenario has been reported for vasopressin translocating IRAP (oxytocinase) to the plasma membrane in renal cells [88]. Treatment of the proximal tubule cell line, NRK52E with vasopressin (10^{-8} to 10^{-6} M) resulted in a significant increase of IRAP at the cell surface an effect that was blocked by a specific V_2 receptor antagonist.

The identification of the high affinity, binding site for the pentapeptide, Angiotensin IV (Ang IV), the AT_4 receptor, as IRAP provides another new functional significance for IRAP [71]. The AT_4 receptor and its modulation by Ang IV have been associated with cellular proliferation, vasodilation and effects in the central nervous system [89]. Infusions into the brain of Ang IV or its stable analogues, Nle^1-Ang IV and Nle^1-$Leual^3$-Ang IV (AT_4 ligands) improve memory retention and retrieval in both normal rats and in rats with memory loss as demonstrated in a range of behavioural paradigms [90, 91, 92, 93]. In keeping with their cognitive effects, AT_4 ligands enhance long-term potentiation (LTP) in both the dentate gyrus *in vivo* [94] and the CA1 region of the hippocampus *in vitro*, [95]. (LTP is a cellular model for memory formation and is a measure of increased synaptic efficacy after high-frequency stimulation). In addition AT_4 ligands also enhance K^+-evoked acetylcholine release from rat hippocampal slices [96] in keeping with the relatively high levels of IRAP found in the septo-hippocampal pathway.

AT_4 receptor ligands, including Ang IV, and the structurally unrelated LVV-hemorphin-7, have recently been demonstrated to all be potent inhibitors of IRAP catalytic activity, as assessed by cleavage of the synthetic substrate, Leu-β-naphthylamide, by recombinant human IRAP [74]. Ang IV displays competitive kinetics indicating that AT_4 ligands mediate their effects by binding to the catalytic site of IRAP. We have recently confirmed these results by demonstrating that mutations of key residues in either the GAMEN or zinc binding motifs significantly alter the affinity of AT_4 ligands for IRAP. Therefore the physiological effects of AT_4 ligands, including enhanced cognition may result from inhibition of IRAP cleavage of peptide hormones.

CONCLUDING COMMENTS

The membrane bound aminopeptidases of the M1 family contain the transmembrane domain near the N-terminus functioning as ectoenzymes and in some cases within specific intracellular compartments. In addition soluble forms of the aminopeptidases are produced by cleavage at the plasma membrane. All appear to form homodimers, and in some cases are covalently linked (eg. APA, TRH-DE). The broad substrate specificity towards naturally occurring peptide hormones observed for the aminopeptidases *in vitro* complicates the understanding of their physiological roles. However the advent of new specific inhibitors and characterisation of *in vivo* substrates will pave the way towards elucidating additional roles for this class of enzymes.

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Progress in the Development of Aminopeptidase N (APN/CD13) Inhibitors

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Abstract: Aminopeptidase N (APN; CD13) is a member of zinc-containing ectoenzymes family involved in the degradation of neutral or basic amino acids (Ala>Phe>Leu>Gly) from N-terminal of bioactive peptides and amide or arylamide derivatives of amino acids. The expression of APN being up regulated has been implicated in the pathogenesis of a variety of diseases such as cancer, leukemia, diabetic nephropathy, and rheumatoid arthritis. Thus, APN inhibitors (APNIs) are expected to be useful for the treatment of these disorders. This article reviews briefly the structure characteristic and possible function of APN. The proposed biomolecular structures and mechanism of action used in the design of APNIs are thoroughly covered. Major emphasis is on recently published potent, small molecular weight APNIs and their essential structure activity relationship (SAR). Finally, available clinical results of compounds in development are summarized in this review.

Key Words: Aminopeptidase N, inhibitors, mechanisms.

1. INTRODUCTION

Aminopeptidase N (APN; EC3.4.11.2; gp150) is a monomeric or homodimeric type II membrane-bound glycoprotein [1], and a member of M1 family of Zn^{2+}-dependent metalloectopeptidase and aminopepitdases [2]. It is widely expressed on the surface of renal and intestinal brush border cells [3], myeloid progenitors and monocytes, synaptic membranes in central nervous system (CNS), fibroblasts, endothelial cells, placenta and tumor cells [4]. The enzyme is identical to human lymphocyte surface cluster differentiation antigen, CD13 [5] and was also shown to be the major receptor for the transmissible gastroenteritis virus (TGEV) [6], which causes a severe gastroenteritis in newborn pigs, and for the human coronavirus 229E (HCV229E) [7], which causes upper respiratory infections. APN is overexpressed on tumor cells, which plays a crucial role in tumor angiogenesis [8]. This ectopeptidase releases an N-terminal amino acid, Xaa-|-Xbb- from a peptide, amide or arylamide. Xaa is the neutral or basic amino acids in particular, preferably Ala, but may be most amino acids including Pro (slow action), which may be the component of extracellular matrix (ECM) [9, 10], facilitating the invasion, growth and metastasis of cancer cells. When a prolyl residue follows a terminal hydrophobic residue, the two may be released as an intact Xaa-Pro dipeptide. Furthermore, APN is also involved in the down-regulation of signal peptides, such as enkephaline in the brain and can cleave bioactive proteins, including several cytokines and antigen delivering peptides, degrading lots of immunoactive substances, impairing the immunological functions, depressing the recognization of macrophage and NK cells to surface antigen on tumor cells and the ability to kill these cells directly. In recent years, APN has been elucidated to participate in the enzymatic cascade of rennin-angiotensin system in the brain and periphery by cleaving angiotensin III to antiotensin IV[11]. APN also has been implicated in the processing and trimming of antigenic peptides that protrude out of major histocompatibility complex (MHC) class II molecules on the cell surface [12, 13]. In the presence of specific stimuli exemplified by cytokines such as interleukin 4 (IL-4) and interferon γ (IFN-γ) and growth factors, APN expression is up-regulated resulting in an excessive degradation of ECM components and immunoactive substances and is believed to contribute to numerous pathological conditions: cancer, leukemia, rheumatoid arthritis [14], angiogenesis [8], diabetic nephropathy [15] and central nervous system diseases such as Alzheimer's disease [16]. Therefore, APN inhibitors (APNI) may prove to be clinically efficacious for the treatment of these disorders.

2. STRUCTURE CHARACTERISTICS AND FUNCTION OF APN

Structure Characteristics

Sequence comparisions and site-directed mutagenesis experiments [17] have suggested that APN have the same mode of zinc coordination as a large group of zinc endopeptidase typified by thermolysin (EC 3.4.24.28, TLN), a bacterial protease that has been crystallized with a variety of different inhibitors [18]. Jongeneel and coworkers [19] claimed that all aminopeptidases of M_1 family contain the *HEXXH* conserved motif and the zinc is coordinated by two histidine residues and one glutamate 18 residues C-terminal to the second histidine, which suggests that the active site of APN may be closer to that of a classical zinc endopeptidase, such as TLN, than to that of an exopeptidase, such as Carboxypeptidase A, which does not contain the above sequence. In addition, chemical modifications of APN have indicated that the general organization of its active site resembles that of TLN with the involvement of Y, R, H, and E/D residues in the hydrolytic activity of the enzyme.

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Moreover, the development of APNIs has shown that the S_1, S_1'and S_2' subsites of this peptidase prefer hydrophobic side chains, as do the corresponding subsites in TLN. However, no crystal structural data on this important class of aminopeptidases is available, and in particular the element responsible for the "amino-terminal" specificity of these enzymes has not been defined.

The genome of APN designated ***ANPEP*** is located on human chromosome 15q25-q26 comprising 20 exons [20], which encode a protein of 963 to 967 amino acid residues, depending on the species. APN is anchored in plasma membrane with a small N-terminal cytoplasmic tail (9-10 amino acids), a 23- or 24-amino acid transmembrane domain, and a large extracellular ectodomain that contains the active site. So it displays its activity extracellularly, just like neutral endopeptidase (NEP, CD10), dipeptidyl peptidase IV (DPPIV, CD26), APA, and serum γ-glutamyl transpeptidase (γ-GT, CD224), unlike Matrix Metalloproteinase (MMP), APN functions as an exopeptidase. For secondary structure of APN, it is suggested by Sjöström and coworkers [21] that APN has a domain structure of seven different domains Fig. (1), which perform different functions.

Domain I is the cytosolic part of APN, which contains nine residues. It has not been possible to ascribe any function to this domain of APN.

Domain II is the membrane-spanning domain probably existing as one α-helix. The domain is well conserved within APNs, and is suggested to form the basis for selective apical transport of newly synthesized APN from the trans-Golgi network.

Domain III has a stalk region between residue 40 and residue 70, and after this residue there is a sequence WNXXRLP in APN, which is homologous to non-membrane bound aminopeptidases. A common feature of all aminopeptidases is the occurrence of polyproline comprising polyproline II-helix structure, having three amino acids per turn. This suggestion provides a strategy for the design to APNIs, that is, to design inhibitors with proline scaffold to resemble the three-dimension structure of APN and its substrates (see APN inhibitors section).

Domain IV is composed of amino acid residue 70 to 252 and residues 216-227 is a conserved region that is indicated to be related to the APN enzymatic activity. However the site-directed mutagenesis experiments performed by Luciani *et al.* have indicated that a conserved aspartic acid located in 225 is not critically involved in hydrolytic mechanism [22].

Domain V and domain VI include amino acids 253-580 corresponding to exons 3 to 10. There locate a glutamic acid 355 (E^{355}) in APN conserved region and the mutation of this residue induce a large decrease in enzyme activity, even led to an almost completely inactive enzyme when it is mutated to A [22]. Furthermore, only the inhibitors of APN as transition state analogs could increase the enzymatic activity dramatically, suggesting that this glutamic acid belongs to the anionic binding site in APN. And the possible three-dimension structure of APN contains at least one anionic binding site and three hydrophobic pockets, that is S_1 pocket in the left of zinc and S_1'and S_2' pocket in the right. And the proposed mechanism of action for APN of the M_1 family derived from that of TLN is illustrated in Fig. (2).

Domain VII constitutes the remaining C-terminal part of the enzyme and includes amino acids 581 to 967 (exons 11-20). This domain has a very high content of predicted α-helices. Though this domain contains four of seven cysteine residues, there are no disulfide bridges between domain VII and the rest of the enzyme. And the final part of APN (residue 936 to 964) is composed of a high amount of charged amino acid, which is suggested to be associated with dimerization of the enzyme, and predicted to constitute a dramatically long α-helix suggested to be located outside of the main part. All these properties together with the high conservation within APN from other species suggest a distinct biological function.

The Structure of Inhibitor Binding Sites

APN is a member of M_1 family of zinc dependent metallo-exopeptidase and belongs to gluzincins subfamily [23, 24], which includes APN, aminopeptidase A (APA), aminopeptidase B (APB), leukotriene A_4 (LTA_4) hydrolase, puromycin-sensitive aminopeptidase (PSA), *E. coli* amino-

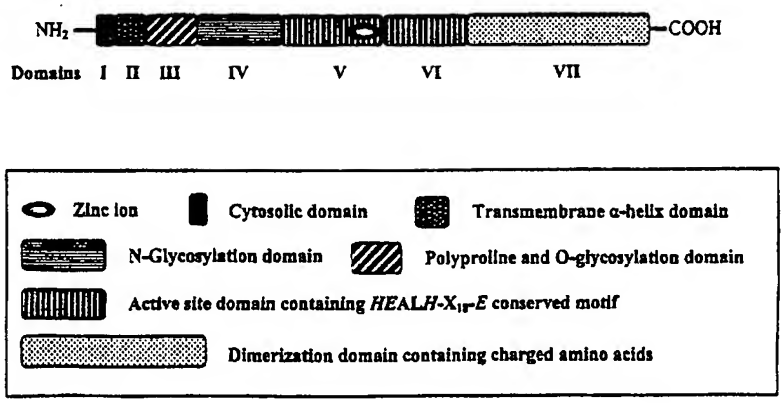


Fig. (1). The proposed domain structures of APN. The lengths of the domains are drawn in proportion to the actual numbers of amino acid residues.

peptidase N and an alanine/arginine aminopeptidase encoded by the *AAP1* gene in *Saccharomyces cerevisiae*, and *Escherichia coli* pepN. In this family the short zincin motif lies in the consensus sequence ***HEXXH***-X_{18}-**E**. The third zinc ligand, glutamic acid, which has been positively identified in LTA_4 hydrolase is present in the consensus sequence LWLN***E***GB which lies a similar distance (18 amino acids residues) on the C-terminal side of the zincin motif as in the TLN family. Sequence analysis of APN has shown that this zinc exopeptidase contains this consensus sequence, generally found at the active site of zinc-dependent metallopeptidases [19]. Hòwever, the nature of the other amino acids involved in the enzymatic activity of APN remains unknown and there is no crystallized structure for APN, though there are a variety of report about other member of aminopeptidase such as Methionine Aminopeptidase (EC 3.4.11.18) [25] and bovine lens leucine aminopeptidase(EC 3.4.11.1, LAP) [26]. However, amino acids residues which are involved in the proposed binding mode of inhibitors or substrates to APN have been elucidated by Grembecka *et al.* including Glu355 (playing a critical role in stabilizing the transition state), His388, Glu389 (coordinated with a water molecule), His392, Glu411 (interacting with zinc ion) and Tyr477 (involved in hydrogen bond with inhibitor) [27]. The protection experiments conducted by Helene and coworkers [28] indicated that functional arginyl and tyrosyl are probably located in the S1'-S2' subsites, and histidine is located in the S_1 subsite and the acidic residue is near the zinc binding site or the S1' subsite. The arginine, histidine and acidic residues are involved in substrate binding, while the tyrosine may play a role in the catalytic process by formation of hydrogen bond and aromatic interactions. LTA4 is the only protein with reasonable sequence identity to APN (27% for most of APN catalytic domain). Gremvecka *et al.* [27] suggested in 2003 that the model of APN binding site is similar to that of LTA4 and bestatin (1) [29].

The affinity of inhibitors with APN was strongly dependent on the nature of the side chain at the P_1'position of inhibitors and weekly on that of P_1 and P_2 position. The S_1' pocket of APN comprises tyrosine and histidine residues, which gives more favorable interactions with aromatic rings than with other side chains because of the increase of hydrophobic contact area and possible interactions between the aromatic rings and the S_1' pocket aromatic residues, which can be exemplified by bestatin (1). The APN active site model also predicts the existence of a hydrogen bond between the hydroxyl group of Tyr present at the P_1' position and the carboxyl group of Glu (anion binding site) located at the bottom of the S1' enzyme pocket.

The model of binding site indicated that peptidomimetics containing aromatic side chains have a significant strong binding affinity toward APN S_1' pocket versus peptide analogues containing aliphatic side chains. Thus incorporation of an aromatic residue at the P_1' site increases the binding affinity toward APN with a simultaneous decrease of specificity toward other enzymes such as LAP. Furthermore, hydroxyl groups substituted on aromatic rings in the P_1' site of the inhibitors will significantly increases the affinity between APN and its inhibitors by formation of hydrogen bond between hydroxyl group and S_1' subsite of the enzyme.

Alternatively, there is a strong need for a free α-amino group or a cation group in presumably the most active APNIs as they probably interact with both the zinc ion and an glutamic acid residue of the active site *via* a electrostatic interaction under its protonated form in physiological conditions.

The Mechanism of Action for APN

There are controversial opinions on the mechanism of action for APN: Bryce [30] believed that the metallic ion is complexed by both the free amino group and carbonyl of the scissile peptide bond. However, Gordon [31] and Digreorio [32] did claim that the free amino group interacts in its proponated form with an anion-binding site, which is composed of Glu or Asp located in the active site of the enzyme, by electrostatic interaction. Taken all the data together, in 1998, Luciani and colleagues [33] put forward the proposed mechanism of action for APN according to that of TLN, which have been studied extensively since 1977 when its crystallied structure complexed with lots of inhibitors was deposited in Protein Data Bank.

The mechanism of action that could be proposed for APN would involve the bidentate coordination of Zn^{2+} by the free amino group and the carbonyl group of the substrate. These two ligands, in addition to the water molecule and the three protein ligands, would lead to a hexa-coordinated zinc complex Fig. (2). There would also be an additional, but slight, interaction between the hydrogen of amino group and Glu355. However, in the transition state it seems highly improbable, for geometric and energetic reasons, that a constrained intermediate having both the hexa-coordination of the Zn^{2+} ion and the tetra-coordination of the peptide-bond carbon would be formed. It seems more probable that the transition state occurs as described for TLN, with the additional creation of stable hydrogen bonds between the free amino group and Glu355, to the detriment of its zinc binding. The strict exopeptidase action of APN and related peptidases does seem therefore to be due to the involvement of the free amino group in the catalytic process Fig. (2). This model could also explain why the affinity for APN decreases significantly when the N-terminal free amino group is absent.

In this mechanism, a simplified model is shown in Fig. (2). The Zn^{2+} is coordinated by carbonyl group of the peptide bond, a water molecule, and the three protein-zinc ligands. In the transition state, a nucleophilic attack of peptide bond by the water molecule, which is polarized by its substrate-induced proximity to the Glu389, leads to a relatively constrained intermediate in which Zn^{2+} is always penta-coordinated and the carbon of the peptide bond is tetrahedral.

The mechanism of action of APN that has emerged from Luciani and coworkers is displayed in Fig. (2). In native APN the zinc ion has approximate tetrahedral coordination, with three ligands provided by the protein (His388, His392, and Glu411) and the forth provided by a water molecule. The incoming substrate is presumed to displace this solvent molecule toward Glu389. Model building suggests that the Michaelis complex is formed when the carbonyl oxygen of the scissile bond is near the zinc. In this complex (Fig. 2a) the carbonyl oxygen and the free amino group are presumed to coordinate with zinc ion. The incoming substrate opti-

mizes its interactions in the S1, S1', and S2' subsites by driving the zinc-bound water molecule toward Glu389. Having both protons hydrogen bonded to Glu389 could enhance the nucleophilicity of this water molecule, and at the same time, having the oxygen liganded to the zinc ion as suggested in Fig. 2a. This tripartite interaction would leave the remaining lone pair directed toward the carbonyl carbon of the substrate and aligned for nucleophilic attack. Under the combined influence of the metal ion and the glutamate, the water attacks the carbonyl carbon to form the pentacoordinate intermediate shown in (Fig. 2b). The proton accepted by Glu389 is then immediately transferred to the leaving nitrogen (Fig. 2c). The collapse of this intermediate (Fig. 2c) to final products (Fig. 2d) is presumed to facilitated by a second proton transfer *via* Glu389. In this case the proton is accepted from the hydrated peptide and shuttled to the leaving nitrogen. However, X-ray crystallographic data of APN or zinc aminopeptidase complexed with inhibitor mimicking the transition state will be necessary to give more details on this proposed recognition mechanism.

Function and Possible role of APN in the Development of Malignant Tumorigenesis

The growth, invasion and metastasis of tumor cells are complicated processes involving proliferation of tumor cells, the degradation of ECM, and angiogenesis. APN plays a critical role in all these processes among which the degradation and neovasculation are of vital importance; meanwhile, the degradation of immunoactive peptides such as interleukin 8 (IL-8) [34] leads to the impairment of body immunosystem, which is in favor of the invasion of tumor cells.

1) Degrading ECM, Promoting the Growth and Metastasis of Primary Tumor

The ECM is a complex structural entity surrounding and supporting cells that are found within mammalian tissues. The ECM is often referred to as the connective tissue that is a natural barrier of invasion of tumor cells. The ECM is composed of three major classes of biomolecules including: *Structural proteins*, such as collagen and elastin; *Specialized proteins*, such as fibrillin, fibronectin, and laminin; *Proteoglycans*, which are composed of a protein core to which is attached long chains of repeating disaccharide units termed of glycosaminoglycans (GAGs) forming extremely complex high molecular weight components of the ECM. Cancers begin as a primary tumor. At some point, however, cells break away from the primary tumor and travel in blood and lymph, thus establishing metastases in other locations of the body. Metastasis is the common cause of death of cancer patients. In order to enter/exit the blood or lymph, cancer cells must pass through a basement membrane, such as ECM.

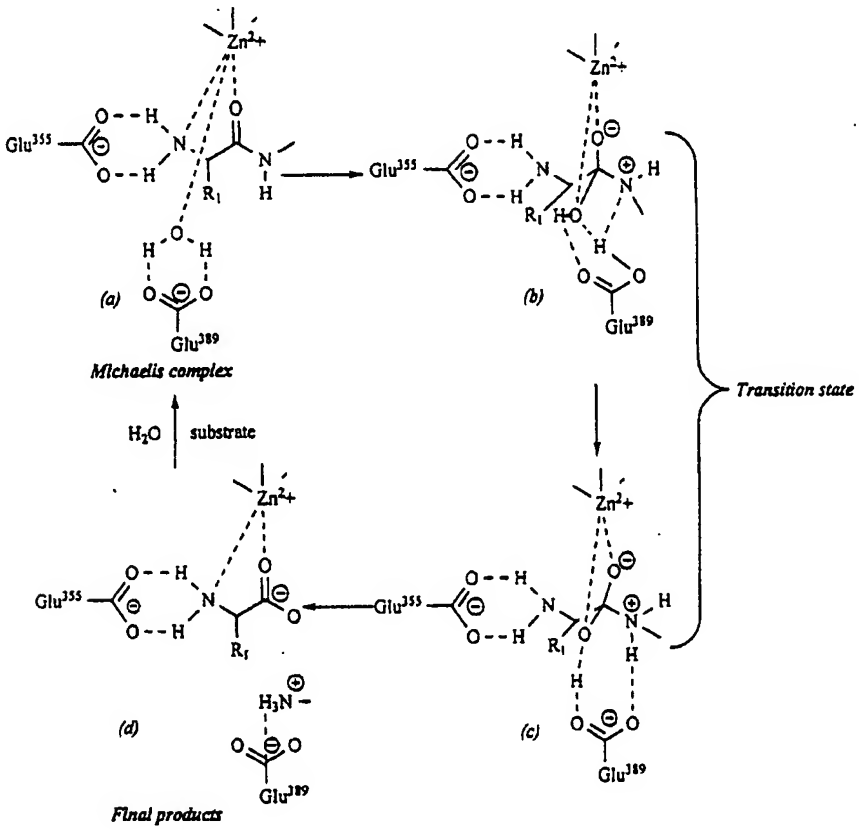


Fig. (2). Schematic depiction of the APN proteolytic mechanism of action for the hydrolysis of a peptide bond and the proposed role of free amino group in the mechanism.

They are able to do so by secreting proteinases (including serine proteases, MMP and APN) that digest a path for them. Saiki *et al.* [9, 10] suggested that APN may be partly involved in the activation mechanism for type-IV collagenolysis to achieve tumor-cell invasion and WM15, a monoclonal antibodies (mAbs) specific for APN, inhibited the invasion of human metastatic tumor cells into Matrigel-coated filters through a mechanism involving its inhibitory action on the APN in tumor cells without any effect on tumor-cell adhesion and migration to the ECM by inhibiting the degradation of type-IV collagen by tumor cells in a concentration-dependent manner. Furthermore, inhibition of APN could prevent the secretion of type IV epithelial collagenase (MMP-9) implicated in collagen degradation [35].

2) Promoting the Angiogenesis of Tumor Tissues

It is suggested that angiogenesis, the formation of new blood vessel, is a rate-limiting step in solid tumor growth. The most common sprouting-type angiogenesis includes following sequential steps: detachment of pre-existing pericytes for vascular destabilization, degradation of ECM by endothelial proteases, such as MMP or APN [36], migration of endothelial cells (ECs), proliferation of ECs, tube formation by ECs, and reattachment of pericytes for vascular stabilization. The phage display experiments conducted by Pasqualini and coworkers [37] indicated that APN is another receptor or marker for tumor-homing peptides and a target for inhibiting angiogenesis except MMP, $\alpha v\beta 3$ integrin and other receptors for vascular growth factor. It is shown that hypoxia is one of the most important triggers of angiogenesis, which induces the expression of vascular endothelial growth factor (VEGF) in various cell types [38]. It was reported that the treatment of animals bearing carcinoma xenografts with APN functiongal inhibitors impaired the tumor growth [9]. And APN was shown to express exclusively in ECs of neo-vessels but not of normal vasculature [37, 39]. Moreover, basic fibroblast growth factor and its downstream Ras-depentent signals mediating EC network formation induced APN expression in ECs [40]. All these observations may indicate that APN expressed on ECs plays a critical role in the regulation of angiogenesis.

3) Degrading Bioactive Peptides and Immunoactive Substance

APN was expressed on the surface of synovial cells, identical to cells derived from rheumatoid and osteoarthritic joints [41]. It has been claimed that APN was responsible for metabolism of its natural substrates, e.g. bradykinin, Leu-enkephalin, substance P together with CD10 and CD26. Tani *et al.* [42] claimed that APN induces chemotattic migration of T lymphocytes by it enzymatic activity and may have a significant role in the pathogenesis of sarcoidosis or alveolitis and in inflamed joints of rheumatoid arthritis patients as a T-lymphocyte chemoattractant. APN was found to degrade immunomodulating peptides (IL-8, tuftsin, thymopentin) and cytokines impairing the normal function of lymphocytes and chemotactic activity, accelerating the invasion of tumor cells. The chemotactic activity of IL-8 was decreased by APN or neutrophil plasma membranes in a dose- and time-dependent manner. The chemotactic activity was not inactivated in the presence of bestatin or WM15 monoclonal antibody. These results suggest that the expression and release of IL-8 from phagocytic cells are regulated by the proteolytic effect of APN on IL-8 [34]. What has been concerned above indicates that APNIs could decrease the degradation of immunoactive substance improving the immunological response of lymphocytes; thereby potentiate the immunological function to combat the invasion and metastasis of tumor cells.

3. NATURAL SUBSTRATES FOR APN

Routine substrates for APN include leucine-p-nitroanilide (leu-pNA), Ala-pNA and Lys-pNA [43]. Purified APN molecule preferentially hydrolyzed Ala-, Leu- and Met-pNA as substrates. Especially, Leu-pNA proved to be the best substrate for APN from larval midgut of silkworm [44].

The type of natural substrates for APN can be divided into four groups: vasoactive peptides (lysyl-bradykinin, angiotensin III), neuropeptide hormones (leu- and meten-kephalin, neurokinin A, somatostatin), cytokines and immunomodulating peptides (IL-8, tuftsin, thymopentin); all of these natural substrates are associated with various physiological or pathological function. Vascular APN hydrolyzed the N-terminus of kallidin to produce bradykinin, and inactivated des(Asp1)angiotensin I, angiotensin III, hepta(5-11) substance P, hexa(6-11)substance P and Met5-enkephalin. APN did not, however, hydrolyze bradykinin, angiotensin I, angiotensin II, saralasin, vasopressin, oxytocin or any form of substance P containing a component of the *RPKP* sequence [45, 46]. Peptide metabolism by APN was optimal at pH 7.0 and was inhibited by bestatin (K_i = 2.2 μM) and amastatin (K_i = 0.025 μM). [47].

4. INHIBITORS FOR APN

One approach for controlling abnormal APN activity is through the use of natural or small molecular weight synthetic inhibitors, which bind directly to the catalytic site of the enzyme. Examples of peptidic and non-peptidic APNIs have proliferated the literature over the last several years, and the first natural inhibitors that have been available in Japan is bestatin (1) for the treatment of leukemia as an immunological regulator.

Several excellent reviews on the design of small molecule inhibitors of APN have been published as well as two detailed reviews concerning the rational design of proteolytic enzyme inhibitors [48, 49]. The principal approachs taken for the design of synthetic APNIs is the substrate-based design method and most molecules designed are transition state analogues. In general, peptidomimetics that incorporate zinc binding groups and P and/or P' side chains to interact with the enzyme subsites have been the most common structural features of inhibitors.

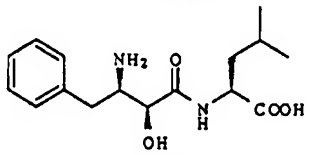
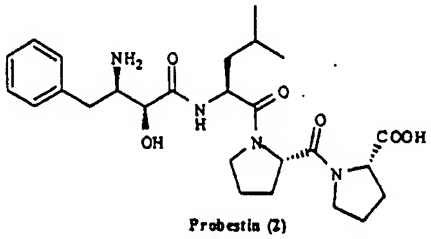
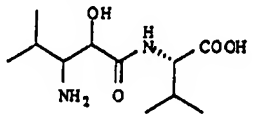
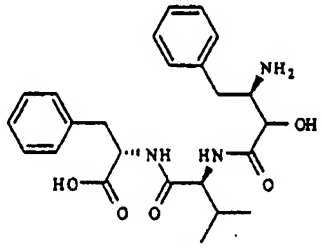
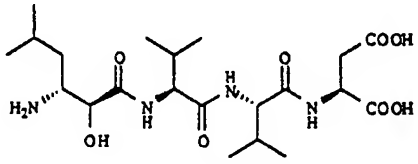
This review will focus on a brief description of small molecule APNIs published in recent years. Small molecular weight inhibitors may be classified into several different structural classes depending upon the structural elements of the inhibitors as well as on the catalytic zinc-binding function. In this review, inhibitors will be grouped into the following arbitrary classifications: (a) natural inhibitors (b) β-amino-Thiols, (c) σ-Aminoaldehydes, (d) Aminophosphonates, (e) β-Amino-α-Hydroxy-Phenyl Butanoic Acid

(AHPA), (f) 4-Amino-L-Proline, (g) L-iso-Glutamines, (h) α-Aminoboronic Acid, (i) Cyclic Imide Skeleton, (j) α-Keto Amide Inhibitors, and (k) 3-amino-2-tetralone derivates. A short description of the rational concepts and SAR used in the design of the individual classes of APNIs is the scope of this review. Interested readers are referred to the many excellent reviews on APNIs design and general reviews of proteolytic enzyme inhibitor design [48, 49] as well as to the references therein.

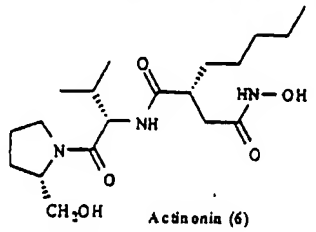
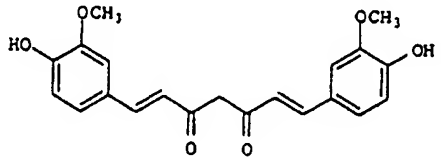
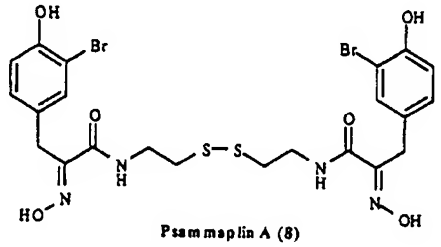
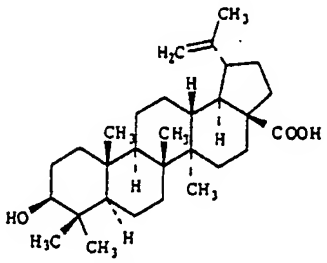
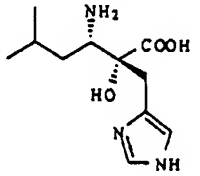
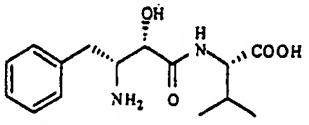
Natural Inhibitors of APN

Some of the most intriguing APNI found to date are natural products. Since 1976, great amount of natural inhibitors have being found to bind APN with high affinity, including bestatin (1), probestin (2), lapstatin (3), phebestin (4), amastatin (5), actinonin (6), curcumin (7), pasmmaplin A (8), betulinic acid (9), leuhistin (10) and AHPA-Val (11), and puromycin(12) and benzo [c] phenanthridine alkaloids (13-15) as well (Table 1).

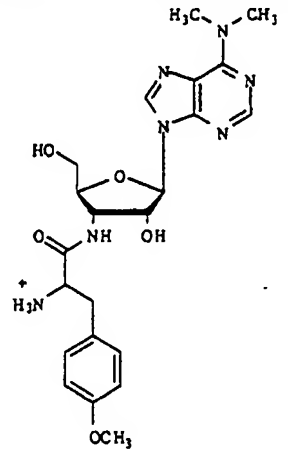
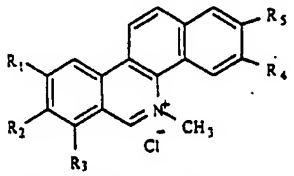
Table 1. Natural Inhibitors of APN

Compd	IC_{50} (μM)	Ref
 Bestatin (1)	IC_{50} (APN)= 16.9 IC_{50} (APW)=7.9 IC_{50} (*P. cuniculi* LAP)=3.9	[50]
 Probestin (2)	IC_{50} (APN)=0.05	[51]
 Lapstatin (3)	IC_{50} (LAP)=0.5	[52]
 Phebestin (4)	Not shown	[53]
 Amastatin (5)	IC_{50} (APA)=1 IC_{50} (pig kinedy LAP)=1.6 IC_{50} (amastatin sensitive aminopeptidase)=9.0	[54, 55]

(Table 1) contd....

Compd	IC_{50} (μM)	Ref
 Actinonin (6)	IC_{50}(APN)=0.2 IC_{50}(hsPDF)=0.043 IC50 (enkephalin-aminopeptidase)=0.39 IC50 (enkephalinase A)=5.6 IC50 (soluble dipeptidylaminopeptidase)=1.1	[56-58]
 Curcumin (7)	IC_{50}(APN of HUVEC)=10 IC_{50} (APN of APN$^+$-HT1080)=7	[59, 60]
 Psammaplin A (8)	IC_{50}(APN)=18	[61]
 Betulinic acid (9)	IC_{50}(APN)=7.3	[62]
 Leuhistin (10)	IC_{50}(*Ascaris suum* amastatin-sensitive aminopeptidase)=1.25	[63]
 AHPA-Val (11)	IC_{50}(porcine kidney microsomal APN)=1.2 IC_{50}(hAPN)=5.6	[64]

(Table 1) contd....

Compd	IC_{50} (μM)	Ref
 Puromycin (12)	Not shown	[65, 66]
 Benzo[c]phenanthridine derivatives Chelerythrine(13) (R_1=H, R_2=R_3=OCH_3, R_4+R_5=OCH_2O) Sanguinarine(14) (R_1=H, R_2+R_3=R_4+R_5=OCH_2O) Fagaronine(15) (R_1=R_2=OCH_3, R_3=H, R_4=OCH_3, R_5=OH)	Not shown	[67]

Bestatin (1) is a dipeptide immunomodulator that is isolated from a culture filtrate of *Streptomyces olivoreticuli.* 1 strongly inhibits APN, APB, LAP, and tripeptidyl and tetrapeptidyl aminopeptidases. It improves immunological function by enhancing the chemotaxis of T lymphocytes and has been known as a potent inhibitor of APN, APB, and LAP in mammalian cells. 1 has a medium skeleton of AHPA as transition state analogues binding with the active site of APN (see Fig. 3). In recent *in vitro* studies, 1 inhibits the invasion of human metastatic tumor cells and induces apoptosis in human non-small lung cancer cell lines. In tumor-bearing mice, it inhibited metastases or tumor growth and prolonged the survival of patients with acute adult nonlymphocytic leukemia who also received chemotherapy and displayed immunomodulatory effect in patients with lymphoma after autologous bone marrow transplantation. A multicneter, double blind, randomized phase III clinical trial of bestatin as patients with completely resected stage I squamous-cell lung carcinoma has just been completed in 2003.

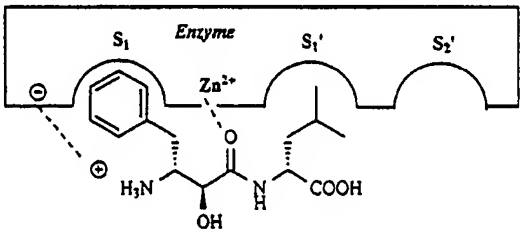


Fig. (3). Schematic representation of the interaction of bestatin (1) with APN.

Probestin (2), Lapstatin (3), Phebestin (4) and Amastatin (5) are all isolated from *Streptomyces* of different categories and are competitive inhibitors of aminopeptidases. Probestin (2) has been isolated as part of a program designed to find microorganism-produced inhibitors of APN with an inhibition constant (Ki) of 19 nM. Lapstatin (3) is the first low-molecular-weight compound shown to inhibit an autogenous aminopeptidase and the extracellular LAP from Streptomyces rimosus, Streptomyces griseus, and Aeromonas proteolytica with an IC_{50} in the range of 0.3-2.4 μM. Phebestin (4) is a new inhibitor of APN with an IC_{50} of 0.015 μM. Like bestatin (1), amastatin (5), a tetrapeptide, also strongly inhibits APN, APA, LAP and tripeptidyl and tetrapeptidyl aminopeptidases and has been used to potentiate the action of some bioactive peptides and manifests subtle selectivity toward aminopeptidases, the inhibition constant of amastatin (5) against human serum APA and pig kidney LAP (Ki) are 1μM and 1.6μM respectively and with no inhibitory activity toward other members of M1 family metalloproteinase, such as APB, thermolysin; chymotrypsin, elastase, and pepsin. Furthermore, it is found that effect of amastatin (5) on angiotensin also evokes neural activity in rat brain.

Actinonin (6) is previously isolated as a pseudopeptide antibiotic that inhibits collagenase at micromolar concentrations and shown to be an inhibitor of APN and LAP. It is discovered that actinonin (6) also has inhibitory activity toward Peptide deformylase (PDF), which is essential in prokaryotes protein synthesis and absent in mammalian cells, thus making it an attractive target for the discovery of novel antibiotics. The dissociation contant for 6 was 0.3 nM against Ni-PDF from *Escherichia coli* and the same value against PDF from *Staphylococcus aureus*. Microbiological evaluation revealed that 6 is a bacteriostatic agent with activity against Gram-positive and fastidious Gram-negative microorganisms [68].

Curcumin (7) is a relatively non-toxic poly-phenolic natural product isolated from the rhizome or underground stem that sends out roots of the leafy plant *Curcuma longa* (turmeric). It consists of two vinyl-guaiacol groups joined by a, β-diketone unit. It has been used in India as a household anti-inflammatory remedy for centuries. In recent years, scientists have discovered many other possible medicinal applications, especially against some kinds of cancers. For the past decade there has been a rapidly growing interest in curcumin's ability to combat breast cancer, carcinoma of colon, intestine, liver, stomach, murine skin and even blood cancer and to slow the progression on Alzheimer's disease because it is safe and can be taken orally. Interestingly, curcumin (7) is also a powerful immune system booster. It may also help promote cancer regression when cancer is present and prevent LDL cholesterol from becoming oxidized. And curcumin (7) is also found to be a modest inhibitor of the HIV-1 (IC_{50} = 100 μM) and HIV-2 (IC_{50} = 250μM) proteases [69]. Furthermore, curcumin (7) and other known APNIs strongly inhibited APN-positive tumor cell invasion and basic fibroblast growth factor-induced angiogenesis. Curcumin did not significantly inhibit the invasion of APN-negative tumor cells, however, suggesting that the anti-invasive activtity of curcumin against tumor cells is attributable to the inhibition of APN. The mode of binding of curcumin to APN has not yet been elucidated. Several studies suggest that α,β-unsaturated ketones of curcumin are critical for the binding to target proteins, and the introduction of various reductive residues including cysteine, dithiothreitol and β-mercapto-ethanol will decrease the activity of curcumin to APN significantly as ketones is reduced to hydroxyl groups, which suggests that these ketones may be covalently linked to nucleophilic amino acid residues in APN's active site.

Psammaplin A (8) is a phenolic natural product isolated from a marine sponge, which showed a potent cytotoxicity against several cancer cell lines. Judging from the structure, Psammaplin A (8) is a tyrosine derivative containing hydroxylamine scaffolds which is a strong binding group to zinc ion. In present study, it was found to have inhibitory activity toward mammalian APN and topoisomerase II with IC_{50} of 18 μM and 18.8 μM respectively and the APN activity was inhibited in a non-competitive manner. Psammaplin A (8) also inhibits bacteria DNA gyrase associated with the growth of certain cell lines. Moreover, it potently inhibits the proliferation of several cancers and suppresses the invasion and tube formation of endothelial cells stimulated by basic fibroblast growth factor endothelial cells and the anti-proliferative effect was dependent on the cellular amount of APN expression. These data demonstrate that 8 is a new inhibitor of APN and can be developed as a novel anti-angiogenic and antibacterial agent. Alternately, Psammaplin A (8) was found to moderately inhibit chitinase B from *Serratia marcescens*, the mode of inhibition being non-competitive. Crystallographic studies suggest that a disordered Psammaplin A molecule is bound near the active site.

Betulinic acid (9), a naturally occurring pentacyclic triterpenoid isolated from Birch trees, is a selective apoptosis-inducing agent that works directly in mitochon-dria. Published data suggest that betulinic acid induces apoptosis in sensitive cells in a P53- and CD95-independent fashion. 9 induces apoptosis in neuroectodermal and epithelial tumor cells and in leukemia cells and enhances TNF-induced apoptosis. Hence, 9 should be further evaluated as a future drug to treat leukemia [70]. In 2003, Takada and colleagues suggested that 9 suppressed NF-κB-dependent reporter gene expression and the production of NF-κB-regulated gene products such as COX-2 and MMP-9 (which are important molecules in inflammation and metastasis of tumor cells) induced by inflammatory stimuli [71]. 9 demonstrated selective cytotoxicity against a number of specific tumor types, a variety of infectious agents such as HIV, malaria and bacteria, and the inflammatory process in general exerting little toxicity in animal trials. Recently, considerable *in vitro* evidence has demonstrated that 9 is effective against small- and non-small-cell lung, ovarian, cervical, head and neck carcinomas. As for the precise molecular target and mechanism of action, accumulated experimental evidence indicates that 9 functions through a mitochondrial-mediated pathway. Supplemental reports suggest that the generation of reactive oxygen species, inhibition of topoisomerase I, activation of the mitogen-activated protein kinase (MAPK) cascade, inhibition of angiogenesis, and modulation of pro-growth transcriptional activators and APN activity may play a role in betulinic acid-induced apoptosis. These potential mechanisms of action may enable 9 to be effective in cells resistant to other chemotherapeutic agents. Arguments

supporting the role of this agent in the treatment of cancers and other infectious conditions have been reviewed [72].

9 inhibits the activity of APN in a dose-dependent manner. An IC_{50} of 7.3 μM was determined for betulinic acid. This inhibitory activity is higher than that of bestatin (IC_{50} = 16.9 μM). The finding supports the idea that betulinic acid acts as anti-melanoma agent *via* inhibition of APN activity.

Leuhistin (10) has been isolated from the culture broth of *Bacillus laterosporus* BMI156-14F1 as part of a program designed to find microorganism-produced inhibitors of APN. Leuhistin (10) inhibits APN strongly and it also inhibits APA and APB weakly. It is competitive with the substrate, and the inhibition constant (Ki) was 0.23μM.

AHPA-Val (11) is a bestatin analogue isolated from the culture filtrate of *Streptomyces neyagawaensis* SL-387 containing 3-amino-3-phenylpropionic acid medium scaffold. 11 was 5 times (IC_{50} = 1.2 μM) stronger than bestatin (IC_{50} = 7.0μM) against porcine kidney microsomal APN, and 3 times (5.6μM) stronger than bestatin (IC_{50} = 20.7μM) against APN of human metastatic fibrosarcoma HT1080 cells.

Synthetic Small Molecule Inhibitors of APN

I. Peptidomimetc inhibitors of APN

Potent APNI known at present are all peptides or non-peptide mimics in chemical structure. Generally speaking, the clinical applications of peptides are limited because of their drawbacks, such as low bioavailability, proteolytic lability, rapid elimination, and short duration of action, etc. From a medicinal chemical point of view, non-peptide mimics are preferable. Studies on the development of inhibitors for zinc-dependent metalloproteinase, i.e., MMPs have mostly been focused on peptidomimetics or peptide mimics which are assumed to act as transition state analogues by binding to the catalytic Zn^{2+} *via* zinc-binding groups (ZBG), such as hydroxamate, carboxylate, sulfhydryl, sulfodiimide and derivatives of phosphoric acid etc. Without exception, the effective APNIs published to date have been designed to incorporate a ligand to bind to Zn^{2+} in the active site of the target enzyme.

1) β-Amino-Thiols Inhibitors of APN [73, 74]

Among the β-Amino-Thiols inhibitors, the ZBG of the APN catalytic site is in the β position with regard to the essential amino group, and very large differences in the inhibitory potencies of these molecules were observed, with the lowest activities being found for the carboxylate and the phosphonate (structure and data not shown) which inhibit the enzyme with IC_{50} in the range of 10^{-4}M. However, the inhibitory efficiencies are enhanced significantly in the existence of hyfroxmate in the same position. The protection of the amino or the thiol function by a benzyl group leads to a large decrease in inhibitory potency, which demonstrates the essential role of the free amino group. The inhibitory potencies of various β-amino thiols differing on by the size and the hydrophobicity of the side chain corresponding to the S_1 pocket of the target enzyme Fig. (4), and the aliphatic groups are preferable compared to aromatic groups (Table 2). The possible reason is the hydrophobic interactions between the substitutent and the S_1 subsite. Ethyl D-cysteinate (27) [74] is a potent competitive inhibitor of APN (IC_{50} = 350 nM) compare to its L-isomer and D-amino acid. The results confirm the hypothesis that the thiol group coordinates to Zn^{2+} at the active site and the alkyl group occupies the hydrophobic binding site for the side chain of the amino-terminal residue of substrates.

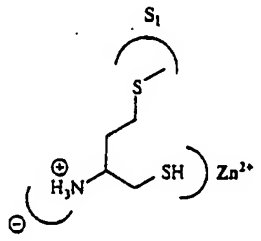


Fig. (4). Schematic representation of interaction between compound 18 and active site of APN.

2) α-Aminoaldehydes Inhibitors of APN [75]

α-Aminoaldehydes analogues prepared by enzymatic oxidation of natural amino acids with alcohol dehydrogenase were also suggested to display inhibitory activity toward aminopeptidases, and L-Leucinal (33) is found to be a very strong competitive inhibitor of microsomes LAP with a IC50 of 0.76μM (Table 3). The unusual affinity of LAP for 33 suggests that inhibition is associated with the specific mechanism of action of this enzyme. And Andersson and colleagues assumed that the Leucinal (33) is bound covalently by aminopeptidase and suggested that this enzyme also acts by a double-displacement mechanism. The free aldehyde group is preferable as it is hydrated when contacted to enzyme and the conformation of aldehyde hydrated resembles "tetrahedral" transition state intermediates of target enzyme.

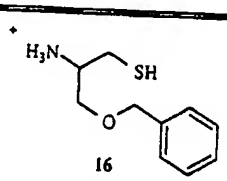
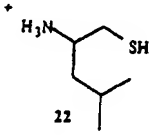
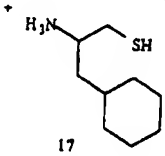
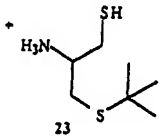
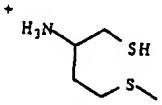
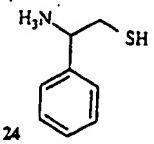
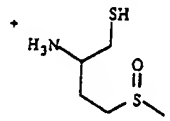
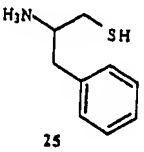
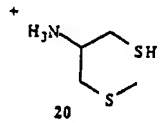
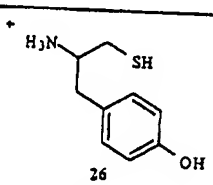
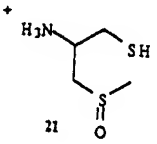
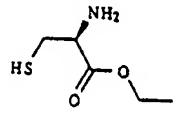
3) α-Aminophosphonates Inhibitors of APN [76-80]

α-Aminophosphonates or α-Aminophosphinic derivatives behave as "transition state" analogues interaction with the S1, S1' and S2', subsites of both NEP and APN (Fig. (5)), and the inhibition constants are in the nanomolar range for the two targeted enzymes (shown in Table 4). Due to their inhibitory activity toward NEP, which plays a critical role in the degradation of enkaphalin, these compounds display analgesic activity. However, because of the presence of three highly polar functions, the passage of these compunds through the blood brain barrier (BBB) is too limited. And the feasible measure to increase their brain penetration is to protect the polar function groups by lipophilic groups transiently to perform prodrug strategy, improving the bio-availability of these compounds.

4) β-Amino-α-Hydroxyphenylbutanoic Acid (AHPA) Inhibitors of APN [81]

Judging from the structure of bestatin (1), AHPA is an active skeleton that binds to the zinc ion and matches the requirement of active site of the enzyme in three-dimension

Table 2. Inhibitory Potency of β-Amino-Thiols Inhibitors on APN Activity

Compd	IC_{50} (nM) APN	Compd	IC_{50} (nM) APN
 16	56	 22	22
 17	45	 23	40
 18	11	 24	25
 19	20	 25	30
 20	20	 26	45
 21	21	 Ethyl D-cysteinate (27)	350

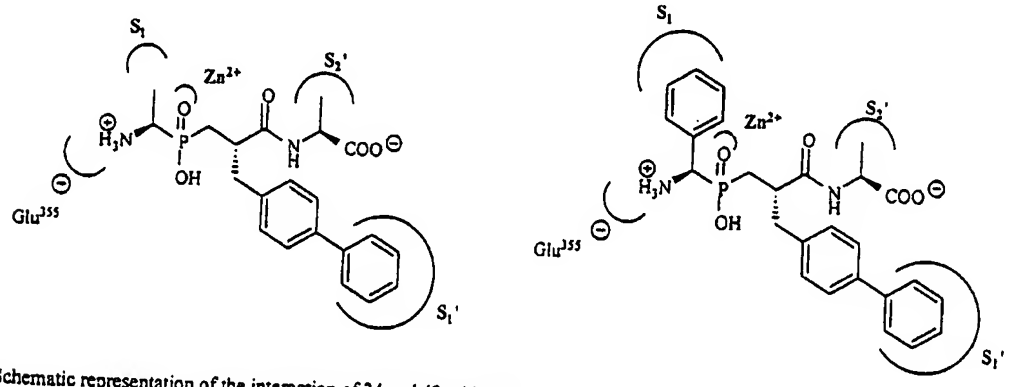


Fig. (5) Schematic representation of the interaction of 34 and 42 with APN.

Table 3. αAminoaldehydes Inhibitors of Microsomal LAP at pH 7.2, 25

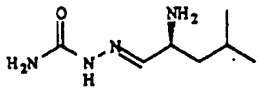
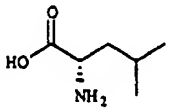
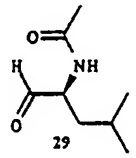
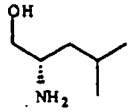
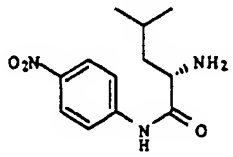
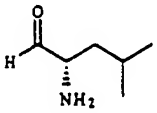
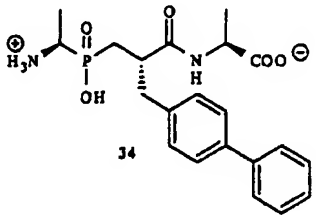
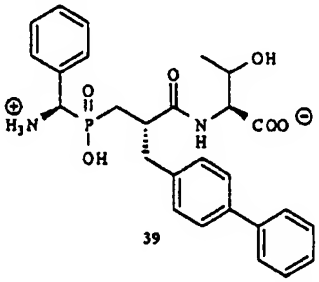
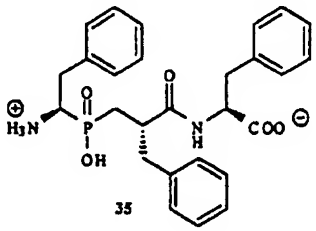
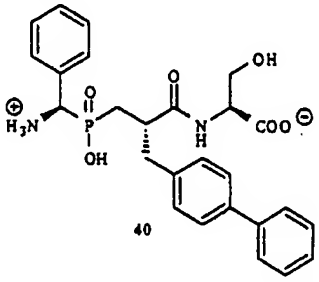
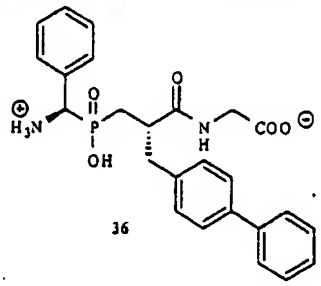
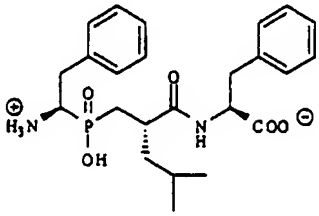
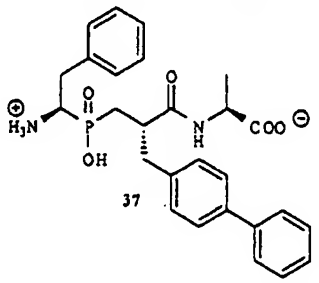
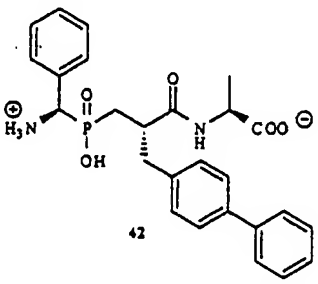
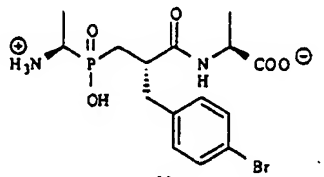
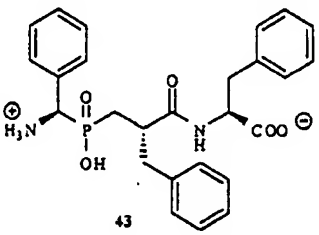
Compd	Ki, (μM)	Compd	Ki, (μM)
 28	230	 31	2950
 29	430	 32	4400
 30	520	 33	0.76

Table 4. α-Aminophosphonates Inhibitors of APN

Compd	Ki (nM)	Compd	Ki (nM)
 34	Ki (APN)=2.9 Ki (NEP)=1.2 Ki (ACE)=120	 39	Ki (APN)=10.2 Ki (NEP)=32.5
 35	Ki (APN)=1.5 Ki (NEP)=190	 40	Ki (APN)=4.9 Ki (NEP)=11.8

(Table 4) contd....

Compd	Ki (nM)	Compd	Ki (nM)
 36	Ki (APN)=32.8 Ki (NEP)=0.94	 41	Ki (APN)=2.3 Ki (NEP)=43
 37	Ki (APN)=5.3 Ki (NEP)=2.2	 42	Ki (APN)=4.8 Ki (NEP)=2.0
 38	Ki (APN)=1.9 Ki (NEP)=4.9	 43	Ki (APN)=4.2 Ki (NEP)=70

structure. However, AHPA is a dipeptide with limitation in clinical application. Allowing for this, we convert the valine residue of bestatin (1) into different amines compounds to obtain potent inhibitors of invasion and metastasis of tumor cells illustrated in Table 5, the inhibitory rate (%) *in vivo* experiments to mouse S180 tumor model metastasis is pretty high.

5) 4-Amino-L-Proline Inhibitors of APN [82]

The function of APN section above has mentioned that APN and MMPs plays a crucial role in the degradation of ECM, the primary component of which is composed of many proline residues. Allowing for this, we designed MMPs inhibitors and APNI with 4-amino-L-proline skeleton to resemble the proline residue of the substrate to compete combining with the enzyme and to inhibit the activity of the enzyme. And evidence of experiment *in vivo* proved that our strategy is correct. Most of the compounds designed have IC50 values in the nanomolar range (Table 6 and 7). Especially, when the methoxyl substitutent on aromatic ring is replaced by hydroxyl group, the inhibitory potency increases significantly, which confirms the importance of hydrogen bond between inhibitors and the active site of the enzyme.

Table 5. β-Amino-α-Hydroxyphenylbutanoic Acid (AHPA) Inhibitors of APN

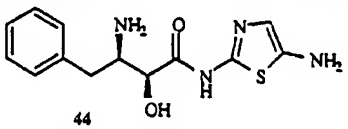
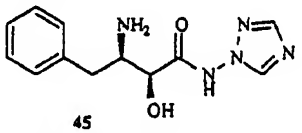
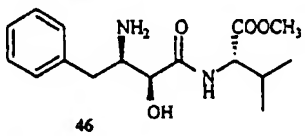
Compd	Inhibitory Rate (%)
 44	37.98
 45	29.92
 46	29.62

Table 6. *Trans*-4-Amino-L-Proline Inhibitors (benzoyl derivatives) of APN

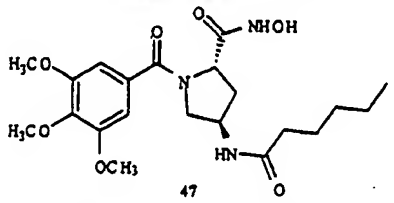
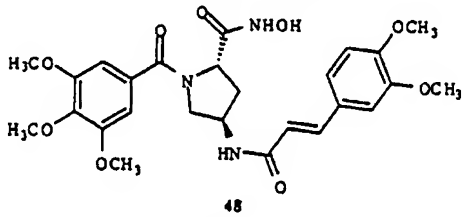
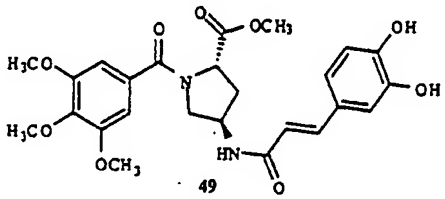
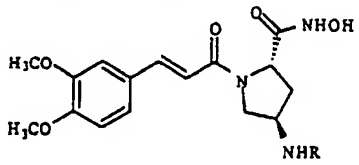
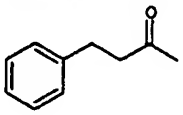
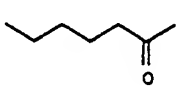
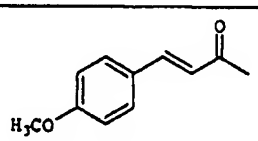
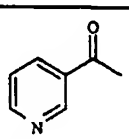
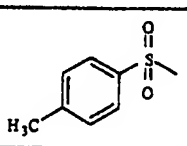
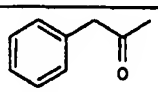
Compd	IC_{50}, nM
 47	7.7
 48	2.1
 49	0.9

Table 7. *Trans*-4-Amino-L-Proline Inhibitors (Cinnamoyl Derivatives) of APN



Compd	R	IC_{50}, nM	Compd	R	IC_{50}, nM
50		15.7	54		9.4
51		7.8	55		6.7
52		9.0	56		9.7
53		11.5			

6) L-iso-Glutamines Inhibitors of APN [83]

Luciani and colleagues [30] put forward that the Glu355 is essential in the formation and stability of transition state intermediates, which inspires a novel thought into us to design a series of APNIs with L-iso-glutamine scaffold. And all of them bear potent inhibitory activity toward metalloproteinase, i.e., APN and MMP-2 and MMP-9 with the IC50 values from 10-200μM (Table 8). In all these compounds, 59 and 65 are most efficient inhibitors toward APN with the IC50 (16.1μM and 22.8μM respectively) proximal to that of bestatin (1) (IC50=13.1μM) because of the presence of hydroxylamate in 59 and hydroxyl group substituted on aromatic ring in 65.

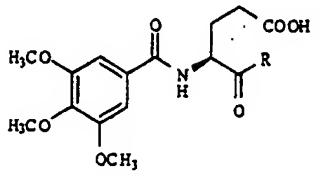
7) α- Aminoboronic Acid Inhibitors of APN [84]

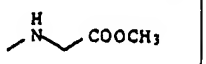
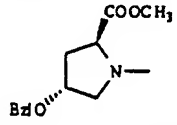
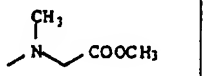
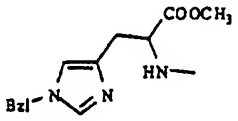
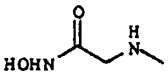
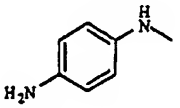
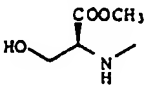
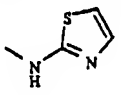
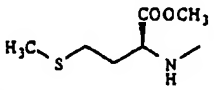
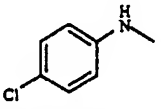
α-Aminoboronic Acid and their derivatives have been demonstrated to be effective inhibitors of proteases, i.e., human enkephalin degrading aminipeptidase, APN, and cytosolic LAP presumably with Ki values in the micromolar range as transition state analogues. These compounds, in which the boron atom has trigonal geometry, can form a tetrahedral boronate ion when interacting with catalytic enzyme. From the kinetics point of view, these compounds exhibit slow-binding kinetics with APN. However, the inhibition appears to be independent of the substituents to the oxygen atoms of the boronic acid (Table 9) and the slow-binding step does not represent the hydrolysis of the borate ester to the free boronic acid. Because of their unique slow-binding inhibitory behavior, α-aminoboronic acids will play a role in the elucidation of the mechanism of action of APN.

II. Non-Peptide Inhibitors of APN

Due to their unacceptable drawbacks, few peptide mimics inhibitors have been performed clinical trials. And researchers turn to the non-peptide mimics' strategy to search perfect APNIs with higher selectivity, higher stability toward protease, and more bioavailability.

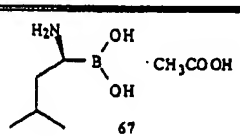
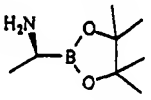
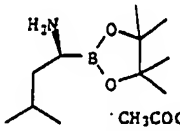
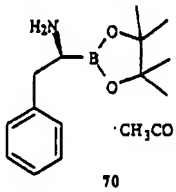
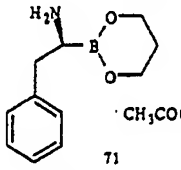
Table 8. L-iso-Glutamines Inhibitors of APN



Compd	R	IC_{50}, μM		Compd	R	IC_{50}, μM	
		APN	MMP-2,9			APN	MMP-2,9
57		53.2	27.4	62		30.2	45.6
58		41.4	nd[b]	63		29.7	38.3
59[a]		16.1	18.9	64		23.4	33.6
60		36.8	72.2	65[c]		22.8	34.6
61		50.7	38.4	66		24.3	26.1

[a] The hydroxyl group of COOH on the mother scaffold is placed by R in compound 59 ; [b] nd=not determined; [c] The methyl residues on the scaffold is replaced by hydrogen atom in compound 65.

Table 9. α- Aminoboronic Acid Inhibitors of APN

Compd	IC_{50}, μM (LAP)	IC_{50}, μM (APN)
 67	0.25	nd*
 68	0.35	0.07
 69	0.25	0.074
 70	0.2	0.05
 71	0.2	nd

*nd=not determined

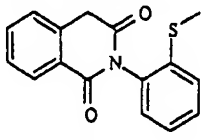
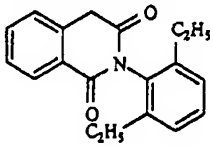
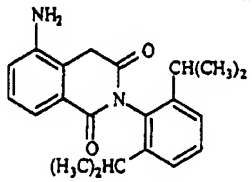
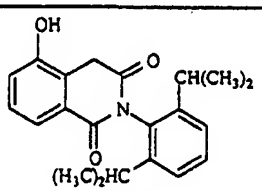
1) Cyclic Imide Skeleton Inhibitors of APN [85, 86]

In 1998, a novel series of small molecule non-peptide APNIs with N-phenylphthalimide or N-phenylhomophthalimide skeleton were synthesized. The molecules with the former skeleton (structure not shown) show potent inhibitory activity toward APN and DPPIV whereas those molecules shown in Table 10 exhibit specific selectivity toward APN (among which compound 74 showed potent tumor-cell invasion inhibitory activity), that is, when the cyclic imide part was changed from a five-membered ring system to a six-membered one, and extraordinary enhancement of APN inhibitory activity was obtained. Furthermore, introduction of an electron-withdrawing nitro group at the fused benzene ring does not affect the activity whereas the compound containing electro-donating group, such as amino (73) and hydroxyl group (75) showed potent APN inhibitory activity.

2) α-Keto Amide Inhibitors of APN

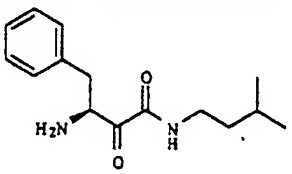
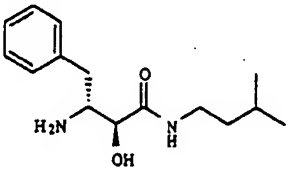
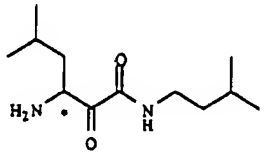
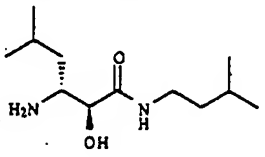
Potent inhibitors of all classes of peptidases have been obtained by replacing the scissile amide bond of a peptidase substrate with carbonyl functionality. Ocain and Rich have reported α-Keto amide inhibitors of APN since 1992, the structure and the mechanism of action of which are similar to bestatin (1) and its analogues. The ketone amino inhibitors may interact with the S_1'-S_2' subsite of the target enzymes, such as cytosolic aminopeptidase, APN, and Argininyl aminopeptidase rather than the S_1-S_1' subsite. And all of them interact with the target enzymes also in a slow-binding manner. The inhibition constant is in the micromolar range and the absolute configuration of photoactive carbon atom has different effect on the inhibitory activity (*S*-configuration is more preferable) and the inhibitory potency is higher in those containing aromatic ring in P_1' site (76 and 77) than those containing aliphatic side chain (78 and 79). However, as can be seen in Table 11, the selectivity toward these APs is unpleasant with the exception of 77 and 79, which are derivatives of AHPA; that is, the carboxyl group proximal to amino group being reduced to hydroxyl group will improving the selectivity toward members of APs.

Table 10. Cyclic Imide Skeleton Inhibitors of APN

Compd	IC_{50}*, (μM)	Compd	IC_{50}*, (μM)
 72	0.90	 74	0.12
 73	5.4	 75	4.3

*The IC_{50} to APN is assayed by the L-Ala-MCA method. The control IC_{50} of bestatin (1) and actinonin (6) are 0.81 μM and 0.32 μM respectively.

Table 11. α-Keto Amide Inhibitors of APN

Compd	Ki, (μM)			Ref
	Cytosolic AP	APN	Arginiinyl AP	
 76	1.0	2.5	1.5	[87]
 77	0.51	20	39	[88]
 78	>15 (R) 1.9 (S)	18.6 (R) 10.5 (S)	6.5 (R) 3.2 (S)	[87]
 79	5.4	24	>300	[88]

3) 3-Amino-2-Tetralone Inhibitors of APN [89]

In France Schalk and colleagues designed a novel APNIs containing 3-amino-2-tetralone skeletons. They are most potent inhibitors to APN with Ki values no more than 1 μM (Table 12). The polycyclic aromatic interacts with the S_1 pocket of the enzyme and the carboxyl group and free amino group coordinate with the catalytic zinc ion (Fig. (6)).

4) Flavone-8-Acetic Acid Derivatives Inhibitors of APN

Novel flavone-8-acetic acid (FAA) derivatives have been first synthesized in 1985 and studied for the treatment of solid tumor since 1993 [92, 93]. FAA derivatives exemplified by 84 proved to be most efficient and exhibited an selective activity toward APN IC_{50} of 25 μM (Table 13), which is 2.5 times higher than that of bestatin (1). However,

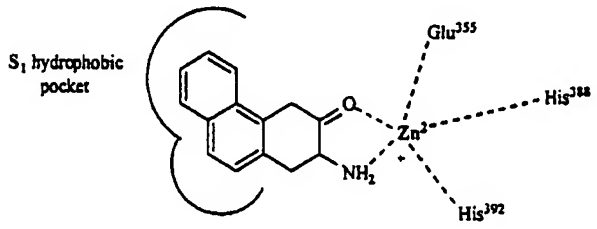
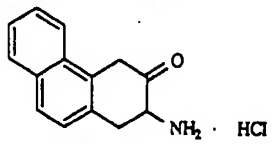
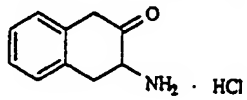
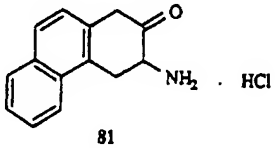
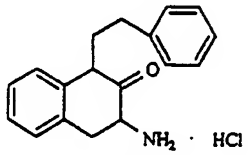


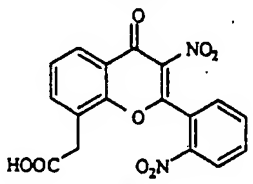
Fig. (6). Schematic representation of interaction of 80 with APN.

Table 12. 3-Amino-2-Tetralone Inhibitors of APN

Compd	Ki[b], (μM)	Compd	Ki[a], (μM)
 80	0.02	 82	0.5
 81	0.2	 83	0.08

[a] All substances are racemic mixtures; [b] Bestatin Ki=3.5 μM, amastatin Ki=0.05 μM

Table 13. Flavone-8-Acetic Acid Derivatives Inhibitor of APN

Compd	IC_{50}, μM	Ref.
 84	IC_{50}(APN)=25	[90-91]

in contrast to bestatin (1), 84 did not induce any cytotoxicity to cultured human model cells. The affinity of 84 for APN is not recovered with other proteases such as MMP-9, angiotensin converting enzyme (ACE, CD143), NEP, γ-GT, or the serine proteases DPPIV.

5. THE ANTICANCER EVALUATION OF APNIS IN CLINICAL TRIALS

Bestatin (1)

Since its appearance bestatin (1) is being used as an immunomodulator for the treatment of many diseases associated with abnormal immunological and haematological function. As an immunomodulatory agent, bestatin has low toxicity even after long-term oral administration and brings about significant modifications in immunological response. In a variety of cooperative randomized controlled study of Bestatin immunotherapy for adult acute nonlymphocytic leukemia [94, 95], prolongation of remission duration and survival was achieved with bestatin immunotherapy combined with remission maintenance chemotherapy. Randomized controlled studies of Bestatin immunotherapy were performed in solid tumors including stage Ib and II malignant melanoma, lung cancer, carcinoma of bladder, stomach, head and neck and esophagus, and therapeutic benefits regarding disease free-interval or survival were observed in certain types of the above-mentioned cancers. Bestatin treatment may enhance the recovery of radiation, postoperative or stem cell transplantation functional defects of the immune system in leukemia of solid tumors patients [96] and has an immunomodulatory effect in patients with lymphoma after autologous bone marrow transplantation [97].

A variety of randomized clinical studies have been performed to evaluate the effects of bestatin to squamous cell carcinoma of the lung. From July 8, 1992 to March 31, 2000, researchs in Japan had been conducting a multicenter, prospective randomized, double-blind, placebo-controlled trial with approximately 400 patients who has their cancer surgically removed involved to evaluate whether postoperative adjuvant treatment with bestatin could prolong the survival of patients with complete resected stage I squamous-cell lung carcinoma. They found that 5-year overall survival and disease-free survival were statistically significantly longer in the bestatin-treated subjects than in the placebo-treated subjects, 81% *vs* 74% and 71% *vs* 62%. The researchers call for additional phase III trials to confirm the result of the current study. This study suggest that the oral administration of bestatin for postoperative adjuvant setting yields a significant improvement on survival and disease free survival in patients with early stage of squamous cell lung cancer [98, 99].

Bestatin is currently available in Japan and three other countries in capsule form for prolonging the survival period after surgery for lung squamous cell carcinoma and has been used as a maintenance treatment for patients with acute nonlymphocytic leukemia who are in complete remission after chemotherapy, as a result of randomized trials for such patients [95, 100]. Up to date, safety assessments of 2164 patients indicate that bestatin is a safe drug with mild and infrequent adverse reactions: the incidence of toxicity of any grade is 1.3% for skin reaction, 1.8% for hepatic dysfunction, and 0.9% for gastrointestinal toxicity including anorexia, nausea, and vomiting.

Bestatin is also evaluated to human immunodeficiency virus (HIV) in many double-blind trials, but the efficiency to HIV is somewhat disappointing. In 1994, the bestatin was discovered to appear to act in the early stages of viral penetration, possibly through inhibition of lymphocyte-associated aminopeptidase [101].

Curcumin (7)

Curcumin is a potent antioxidant with specific antiviral, anti-inflammatory, anti-cancer and cholesterol-lowering effects and a potent chemopreventive agent that has been entered into phase I clinical trials for cancer chemoprevention by the National Cancer Institute, NIH, Bethesda, MD [102]. Many other groups also reported phase I clinical trials of curcumin and showed promising results, i.e. curcumin is pharmacologically safe and has enormous potential in prevention and therapy of cancer [103, 104]. In 2003, curcumin is identified as an irreversible inhibitor of APN. The direct interaction between curcumin and APN was confirmed by *in vitro* and *in vivo* assays.

Curcumin is a potent inhibitor of HIV growth. Unfortunately, in a few small clinical trials, dietary supplementation with turmeric, even at high doses, didn't seem to make a difference with respect to CD4 counts or symptoms of HIV.

Phase I Study of Curcumin sponsored by University of Michigan Comprehensive Cancer Center for the Chemoprevention of Colon Cancer is recruiting patients to determine the dose amount of curcumin that can be tolerated to help in preventing colon cancer in healthy men and women.

On April 19, 2004, curcumin was found by Claudia Colombrita, PhD to be able to help protect the brain against Alzheimer's disease with small doses, at least that's the effect in rats. How it translates to humans has yet to be determined. Curcumin triggers an enzyme known as heme-oxygenase-1 (HO-1), which protects cells from free radical damage that causes inflammation and tissue damage-the root of various diseases such as heart disease, diabetes, and Alzheimer's disease. While her results appear promising, much more study is needed to determine levels of curcumin that could protect against Alzheimer's disease.

6. CONCLUSION

Cancer is one of the most common causes of death in the advanced countries. Approximately one in five people will die of this disease, mostly due to metastasis of tumor cells to other tissues or organs. To date, the primary treatments rely on chemotherapy and radiotherapy to retard tumor growth and metastasis, but these approaches often result in unacceptable side effects. Thus, the concept that APNIs can offer anti-cancer therapy by limiting angiogenesis without direct killing of tumor cells and enhancing the immunological responses of lymphocytes generates enormous excitement among scientists in basic and clinical research as well as in pharmaceutical companies. Furthermore, APNIs may have therapeutic potential towards other unmet medical needs such as diabetic nephropathy, rheumatoid arthritis and viral infections. Hence, the overwhelming trend of successful research and development of APNIs is envisaged. The fact that few compounds have reached advanced clinical trials, despite extensive efforts spent by almost all major pharmaceutical companies, strongly indicates that the development of APNIs is very challenging. Indeed, from the point of view of novel drugs R&D, there is still much work to be done. For example, there is no information about NMR spectroscopy and X-ray crystallography structure of APN. At present, almost all sorts of APNIs designed are transition state analogues, that is, the strategy to design APNIs now is substrate-based design and the SAR studies only revolved around the proposed amino acids located in the active site of the target proteins. Secondly, the concept of inhibiting angiogenesis for the treatment of solid tumor is not proven clinically. Moreover, effective antiviral drugs to combat upper respiratory infection are needed. In 2003, Kontoyiannis and colleagues [105] suggest that the receptor for the SARS coronavirus is human aminopeptidase N (hAPN) or CD13 based on Yeager's work showing that hAPN is a receptor for human coronavirus 229E which causes upper respiratory infection. Afterward, however, Yeager [106] denied what Kontoyiannis's had suggested and claimed that it is not reasonable to use bestatin(1) to prevent or treat SARS since APN has not been established as a cellular receptor for SARS coronavirus, and since competitive inhibitors of APN, either bestatin (1) or actinonin (6), do not inhibit human coronavirus 229E recognition of its APN receptor. In spite of these concerns, enthusiasm in the development of APNIs remains high, and the therapeutic potential is expected to grow when positive clinical data are unveiled.

ABBREVIATIONS

ACE	= Angiotensin converting enzyme
AHPA	= β-Amino-α-Hydroxy-Phenyl Butanoic Acid
APA	= Aminopeptidase A
APB	= Aminopeptidase B
APN	= Aminopeptidase N
APNIs	= Aminopeptidase N inhibitors
APs	= Aminopeptidases
APW	= Aminopeptidase W
BBB	= Blood brain barrier
CNS	= Central nervous system
COX-2	= Cyclooxygenase 2
DPPIV	= Dipepidylpeptidase IV
ECM	= Extracellular Matrix

ECs = Endothelial cells
γ-GT = γ-glutamyl transpeptidase
GAGs = Glycosaminoglycans
hAPN = Human aminopeptidase N
HCV229E = Human coronavirus 229E
HUVEC = Human umbilical vein endothelial cells
IFN-γ = Interferon γ
IL-4 = Interleukin-4
IL-8 = Interleukin-8
LAP = Leucine aminopeptidase
LAT4 = Leukotriene A_4
mAbs = Monoclonal antibodies
MAPK = Mitogen-activated protein kinase
MHC = Major histocompatibility complex
MMPs = Matrix metalloproteinase
NEP = Neural endopeptidae
PDFs = Peptide deformylases
PSA = Puromycin-sensitive aminopeptidase
R&D = Research and development
SAR = Structure activity relationship
SARS = Severe acute respiratory syndrome
TGEV = Transmissible gastroenteritis virus
TLN = Thermolysin
VEGF = Vascular endothelial growth factor
ZBG = Zinc binding group

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